STRATEGIES TO PREVENT GERM CELL DEATH FOR FERTILITY PRESERVATION

Marjut Otala

Program for Developmental and Reproductive Biology
Biomedicum Helsinki
University of Helsinki
Finland

and

Pediatric Graduate School
Hospital for Children and Adolescents
Helsinki University Central Hospital
University of Helsinki
Finland

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Supervisor

Professor Leo Dunkel
Hospital for Children and Adolescents
University of Helsinki
Helsinki, Finland
and
Department of Pediatrics
University of Kuopio
Kuopio, Finland

Reviewers

Docent Olli Ritvos
Program for Developmental and Reproductive Biology
Biomedicum Helsinki
and
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Helsinki, Finland

and

Docent Oskari Heikinheimo
Department of Obstetrics and Gynecology
University of Helsinki
Helsinki, Finland

Official Opponent

Professor Outi Hovatta
Department of Clinical Sciences
Karolinska Institute
Karolinska University Hospital Huddinge
Stockholm, Sweden

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To my Family

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by roman numerals.

I **Otala M**, Erkkilä K, Tuuri T, Sjöberg J, Suomalainen L, Suikkari AM, Pentikäinen V, Dunkel L. Cell death and its suppression in human ovarian tissue culture. Mol Hum Reprod. 2002 Mar;8(3):228-36.

II **Otala M**, Mäkinen S, Tuuri T, Sjöberg J, Pentikäinen V, Matikainen T and Dunkel L. Effects of testosterone, dihydrotestosterone and 17β-estradiol on human ovarian tissue survival in culture. Fertil Steril. 2004 Oct;82 Suppl 3:1077-85.

III **Otala M**, Pentikäinen MO, Matikainen T, Suomalainen L, Hakala JK, Perez GI, Tenhunen M, Erkkilä K, Kovanen P, Parvinen M and Dunkel L. Effects of acid sphingomyelinase deficiency on male germ cell development and programmed cell death. Biol Reprod. 2004 Sep 15 [Epub ahead of print].

IV **Otala M***, Suomalainen L*, Pentikäinen MO, Kovanen P, Tenhunen M, Erkkilä K, Toppari J, Dunkel L. Protection from radiation-induced male germ cell loss by sphingosine-1-phosphate. Biol Reprod. 2004 Mar;70(3):759-67. Epub 2003 Nov 12.

*Shared first authorship.

 In addition, some unpublished data are presented.

ABBREVIATIONS

AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic-protease-activating-factor-1
AR	Androgen receptor
ASM	Acid sphingomyelinase
ASMKO	Acid sphingomyelinase knock-out
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
cAMP	Cyclic adenosine monophosphate
cFLIP	Cellular FLICE-like inhibitory protein
cGMP	Cyclic guanosine monophosphate
DHT	Dihydrotestosterone
EDG	Endothelial differentiation gene
EM	Electron microscopy
ER	Estrogen receptor
FasL	Fas ligand
FSH	Follicle stimulating hormone
GDF-9	Growth and differentiation factor-9
GnRH	Gonadotrophin-releasing hormone
Htr2/Omi	5-hydroxytryptamine (serotonin) receptor 2A/Omi
IAP	Inhibitor of apoptosis protein
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
KO	Knock-out
LH	Luteinizing hormone
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear factor-κB
NSM	Neutral sphingomyelinase
PCO	Polycyctic ovaries
POF	Premature ovarian failure
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SHBG	Sex hormone binding globulin
SM	Sphingomyelin
Smac/DIABLO	Second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI
TNFα	Tumor necrosis factor α
TNF-R	Tumor necrosis factor receptor
WT	Wild type
zVAD-fmk	Benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone

ABSTRACT

Life expectancy of cancer patients has recently improved dramatically. However, both female and male germ cells are sensitive to aggressive cancer treatments, such as irradiation, which may lead to immediate or prospective loss of fertility. Banking of mature germ cells prior to cancer treatment is currently feasible, but not helpful for pediatric patients, who do not possess mature gametes. Gonadal tissue or immature germ cells from children may be cryopreserved for future infertility treatments, but the germ cells have to be matured in order to become competent for production of embryos. *In vivo* maturation of germ cells within transplants of gonadal tissue may not be acceptable for many cured cancer patients, as grafts may contain malignant cells that can cause relapse of the disease.

For females, complete *in vitro* maturation of primordial follicles, the most common developmental stage of the ovarian follicle, has been achieved in the mouse [1, 2], but not in any other mammalian species. Another alternative would be to protect the gonads *in situ* from cancer treatments. The acid sphingomyelinase (ASM)-mediated apoptosis pathway is important for female germ cell death [3]. Accordingly, successful *in situ* protection of mouse ovaries from irradiation-induced damage has been achieved with the lipid second messenger sphingosine-1-phosphate (S1P) [3]. S1P protection of human ovaries is currently under investigation.

For males, complete *in vitro* development of spermatozoa from spermatogonial stem cells has not been successful in any animal species. Additionally, no effective *in vivo* protectants against irradiation-induced death of spermatogonia exist. Thus, there is a growing need to develop methods for *in vitro* maturation and *in situ* protection of male germ cells.

The aim of the first part of this thesis was to characterize the pathway by which ovarian tissue dies under culture conditions and to investigate whether this cell death can be manipulated. We attempted to improve the viability of the tissue with an antioxidant N-acetyl-L-cysteine (NAC) or with the endogenous steroid hormones testosterone, dihydrotestosterone (DHT) and 17β-estradiol. The experiments revealed that survival of human ovarian tissue *in vitro* is hampered by redundant apoptosis in the tissue, especially in the interstitium. Demise of the stroma is followed by death of the follicles. Ovarian tissue apoptosis can be suppressed by NAC (32% at 100 mmol/l) and DHT (37% at 10^{-7} mol/l), but not by 17β-estradiol. Marginal suppression of tissue apoptosis was also obtained with testosterone (26% at 10^{-9} mol/l).

The second part of the thesis aimed at investigating the possibility of manipulating male germ cell survival and death. More specifically, we studied the role of the sphingomyelin (SM) pathway in male germ cell apoptosis. We then attempted to pharmacologically prevent irradiation-induced germ cell apoptosis *in vivo* with S1P. Irradiation induced apoptosis in the mouse male germ cells dose dependently. Studies on male mice deficient in ASM revealed that this enzyme is not required for physiological germ cell death or apoptosis induced *in vivo* by irradiation or by *in vitro* culture. ASM is also dispensable for ceramide production in male germ cells subjected to *in vitro* culture. However, irradiation-induced apoptosis was partially inhibited by intratesticular injections of the rheostat for ceramide, S1P (16%-47%).

In conclusion, the present studies revealed that rapid apoptosis occurs in human ovarian tissue cultures and that antioxidants or endogenous androgens may be applicable survival factors for cultured tissue. Unlike in females, the enzyme ASM is not required for physiological or induced

male germ cell death, or for ceramide production, although S1P is able to partially protect male germ cells from irradiation-induced apoptosis. Thus, the SM pathway does not seem to be the principal initial transduction pathway of male germ cell apoptosis but may serve as an alternative route to cell death. Male and female gametes therefore seem to utilize different primary apoptotic pathways.

INTRODUCTION

Over the past three decades remarkable progress has occurred in childhood cancer treatments. Today, approximately 75-80% of children with cancer will be alive five years from diagnosis [4]. In Finland approximately 130 children will become affected with cancer each year [5]. It has been estimated that by the year 2010 around one in 250-715 adults will be a childhood cancer survivor [4, 6].

Studying the menstrual histories of more than 1000 women receiving treatment against malignancy in adolescence has revealed a 42% incidence in menopause by the age of 31 years, compared with 5% of controls [7]. A gross estimation of the incidence of infertility with azoospermia (no sperm present in the ejaculate) in young male cancer survivors is 16-28% [8]. Thus, the long-term effects of cancer treatments on future reproductive function have become an issue of great concern.

Germ cells are sensitive to irradiation. The LD_{50} radiation dose for human oocyte is <2 Gy [9]. Even if menstrual cycling begins at puberty, exposure to irradiation may accelerate germ cell depletion and expedite the time of menopause. Damage to spermatogonia already occurs after 0.1 Gy, resulting in oligozoospermia (clinically relevant reduction in the number of sperm) in adult men, and doses exceeding 2-3 Gy often lead to irreversible germ cell loss [10, 11]. Many chemotherapy agents are also detrimental to germ cells [12, 13].

Candidate conditions for fertility preservation currently include gonadotoxic treatments also for other systemic diseases and non-cancer conditions [14] (Table 1). Moreover, patients with certain genetic conditions may face gonadal failure and infertility. For example in Turner's syndrome and Klinefelter's syndrome accelerated germ cell loss is often detected before or at adolescence [15, 16] and infertility among these patients is common. Turner's syndrome and Klinefelter's syndrome are relatively common chromosomal disorders, affecting approximately 1 in 2000 females and 1 in 500-1000 male births, respectively [17, 18].

Table 1. Candidate disease conditions for fertility preservation. Modified from Oktay and Buyuk (2004) [14].

Risk of subfertility	**Diseases with gonadotoxic treatments**
HIGH	Bone marrow transplant patients (total body irradiation or chemotherapy conditioning) e.g.cancers: leukemia anemias: aplastic, sickle cell, thalassaemia autoimmune and immune-deficiency diseases: rheumatoid arthritis, SCID Localised radiotherapy: pelvic/testicular Hodgkin's disease: alkylating agent based therapy Soft tissue sarcoma: metastatic Prophylactic oophorectomy e.g.BRCA1 or BRCA2 mutation carriers
MODERATE/LOW	Hodgkin's lymphoma: "alternating therapy" Non-Hodgkin's lymphoma Osteosarcoma Ewing's sarcoma Wilm's tumor Acute myeloblastic leukemia Neuroblastoma Soft tissue sarcoma Benign gonadal tissue tumors Brain tumor: craniospinal radiotherapy (cranial irradiation > 24 Gy) Autoimmune diseases e.g.acute glomerulonephritis, Bechet's disease
Risk of subfertility	**Diseases or conditions affecting fertility**
HIGH	Genetic disorders: e.g.Turner's syndrome (45, X0) Klinefelter's syndrome (47, XXY)
MODERATE/LOW	Genetic disorders: e.g.carriers of fragile-X syndrome Autoimmune diseases

Since the birth of the first baby conceived *in vitro* in 1978 [19] infertility treatments have greatly improved. *In vitro* fertilization (IVF) procedures have reached pregnancy rates of almost 30% per embryo transfer [20]. In cases where future infertility can be expected, an effective way to preserve reproductive potential is the production and cryopreservation of embryos. This approach, however, may be impossible due to lack of a spouse or acuteness of disease. For females, cryopreservation of oocytes has recently become possible and some pregnancies have been achieved [21, 22]. For males, a well-established method to preserve fertility is cryopreservation of sperm [23]. However, none of these methods are feasible for prepubertal children lacking mature haploid gametes. The ovaries of young girls are quiescent, endowed with primordial follicles and few growing follicles that will inevitably face atresia [24]. In prepubertal boys active spermatogenesis does not occur [25, 26] hence spermatozoa are absent. To preserve germ cells from children, immature gametes or gonadal tissue must be stored frozen for several years, in some cases for decades, after which the germ cells must be matured. At present ovarian cortical tissue and testicular tissue cryopreservation are feasible [27].

Autografting of gonadal tissue or germ cells cryopreserved before gonadotoxic treatments may later allow maturation of the immature gametes. This approach, however, may not be appropriate for many cancer patients, especially those with hematological malignancies (e.g. leukemia), systemic cancers (e.g. lymphoma), metastasizing malignancies or ovarian/testicular cancer, as malignant cells may be transmitted in grafts [28, 29]. Thus, it is expected that future technologies will enable the *in vitro* maturation of germ cells.

Thus far, full *in vitro* maturation of human primordial follicles into mature antral follicles has not been achieved due to follicular atresia under culture conditions [30-33]. Autologous ovarian tissue grafts have brought about menstrual cycles, ovulation and recently even a pregnancy [34]. This approach could potentially be an alternative for those who are not at risk of transmission of cancer cells (e.g. Turner women). Heterotopic transplantation, i.e. to a site other than the ovarian pedicle, has recently produced a normal oocyte that was fertilized and developed into a four-cell embryo [35]. Protection of the ovaries *in situ* from cancer treatments has been attempted through administration of gonadotrophin-releasing hormone (GnRH) analogs, but despite promising preliminary results clinical studies have been inconclusive [36, 37]. Recently it was shown that a lipid second messenger sphingosine-1-phosphate (S1P) completely protects mouse ovarian follicles *in vivo* from irradiation-induced follicular apoptosis when administered into the ovarian bursa before irradiation [3]. This finding has raised hope that S1P may become a new approach to preserve the oocytes of cancer patients. Moreover, it also showed that manipulation of apoptotic pathways may be effective in *in situ* oocyte preservation.

For male cancer patients, transplantation of germ cells back into the testis where they are able to recolonize tubules may not be safe enough for clinical use [38]. Thus, it might be an option only for conditions that do not involve the risk of re-introducing cancer. Other alternatives for male fertility preservation, such as protecting the germ cells *in vivo*, should be sought. *In vivo* protection of human germ cells from excessive apoptosis induced by cancer treatments is an intriguing alternative. Hormonal prevention of gonadal toxicity of cytotoxic treatments *in vivo* has been ineffective. *In vitro* maturation of germ cells to developmental stages at which they are able to fertilize by intracytoplasmic sperm injection (ICSI) would insure that no malignant cells are transferred [39]. These techniques, however, are currently only experimental and no pregnancies have been attained.

Multiple strategies exist for the protection of germ cells from cytotoxic treatments. However, it is currently not known which strategies will prove most efficient. The first part of this thesis project aimed at improving human ovarian tissue cultures. Survival of ovarian tissue and follicles in cultures is very species-specific. Human follicles grow more slowly and become considerably larger than, for example, murine follicles [40] and therefore most likely have distinct requirements in culture. Data obtained from tissue cultures of other species cannot be extrapolated directly to human ovarian tissue, which makes it essential to use rarely obtainable and highly valuable healthy human tissue in experiments. The second part aimed at inhibiting irradiation-induced male germ cell death pharmacologically *in vivo*. In the *in vivo* experiments, mice were used due to the highly experimental and somewhat invasive nature of the study.

REVIEW OF LITERATURE

Apoptotic cell death

Cell death is a part of normal development as well as a response to endogenous modulation, such as inflammation, and to xenobiotic agents (i.e. micro-organisms and chemicals). It is also an important variable in development of cancer and in its treatment. The classic ultrastructural studies of Kerr et al. (1972) [41] suggested that at least two types of cell death exist. The first type, known as necrosis, involves quick and violent death of cell populations. It is characterized by swelling of the cell, destruction of organelles, plasma membrane disruption and uncontrolled release of intracellular contents, leading to inflammation. The other type of cell death, apoptosis, has distinct morphologic features that are usually expressed in individual cells surrounded by healthy looking neighbors. The dying cells are characterized by cellular shrinkage, blebbing of the plasma membrane, maintenance of organelle integrity, condensation, margination and fragmentation of the nuclear chromatin, cytoplasmic vacuolization and ordered removal of the cell through phagocytosis, and progress without an inflammatory response [41].

Following the characterization of cell death, a dichotomy has prevailed in the literature concerning cell death. Today, however, the line between apoptosis and necrosis is considered less clear cut. Accumulating evidence points to multiple alternative death pathways as well as to cross-talk between the mechanisms involved in distinct aspects of cell death. Although apoptosis is often called programmed cell death this appellation is misleading, as besides apoptosis other forms of cell death, including necrosis, may be programmed and utilize parts of the apoptotic cell death machinery [42]. Classic apoptosis and necrosis have thus been proposed to represent two extremes of a continuum of intermediate forms of cell death designated as aponecrosis (or necrapoptosis) [43].

Apoptotic cell death can be triggered by several stimuli, including receptor-mediated signaling and external or internal insults that create cellular stress. The signals feed into the intracellular execution machinery through a death receptor pathway (i.e. extrinsic pathway) or through the mitochondrial pathway (i.e. intrinsic pathway) (Figure 1) [44]. Both pathways lead to activation of caspases, a family of cysteine proteases. Caspase activation amplifies the apoptosis machinery that demolishes the cell. The morphological features of apoptosis are most often caused by the action of caspases. However, apoptotic cell death does not necessarily involve caspases.

Both the ovary and the testis undergo constant modulation driven by apoptosis [45, 46]. The description of apoptosis routes presented below concentrates on the principal features of the main apoptotic pathways and on the apoptotic events central to the current thesis project.

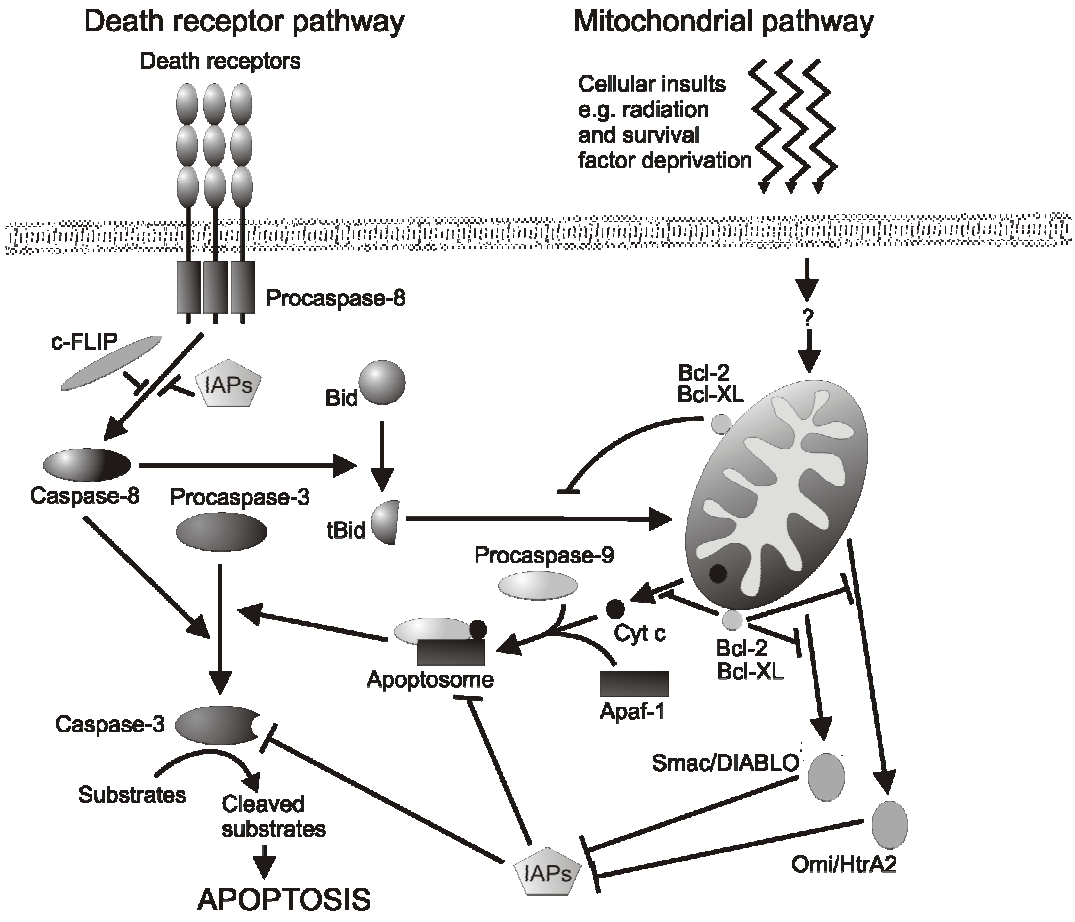

Figure 1. Simplified presentation of death receptor and mitochondrial apoptosis pathways. Death receptor ligands (such as FasL and TNFα) can trigger the death receptor pathway of apoptosis by binding to their receptors, resulting in activation of caspase-8 and subsequent activation of downstream caspases without the involvement of mitochondria. However, through formation of tBid the mitochondrial pathway can be engaged. The degenerate caspase homologue c-FLIP can block caspase-8 activation. Various external stimuli (such as radiation and deprivation of survival factors) can initiate the mitochondrial pathway of apoptosis by triggering the release of mitochondrial apoptotic proteins including cytochrome c (Cyt c), AIF, endonuclease G (Endo G), Smac/DIABLO and Omi/HtrA2. Cyt c binds to Apaf-1 and allows it to bind and activate procaspase-9. The death receptor and mitochondrial pathways converge at the level of caspase-3 activation. Smac/DIABLO and Omi/HtrA2 are able to neutralize IAP-induced inhibition of caspases. Bcl-2-like survival factors such as Bcl-2 and Bcl-XL block apoptosis signaling at the level of mitochondria. Degradation of cellular structures by caspase-3 as well as other downstream caspases and enzymes activated by caspases leads to the typical cellular manifestations of apoptosis, including internucleosomal DNA fragmentation. Either one or both pathways can be simultaneously activated in response to an apoptotic stimulus in a single cell.

Death receptor pathway

Death receptors, i.e. certain members of the TNF receptor superfamily, are located in the cell plasma membrane and possess death domains in their cytoplasmic tails. Once activated by ligands, the death domains recruit adaptor molecules that activate caspases. The well characterized death

receptors Fas (Apo-1 or CD95) and tumor-necrosis factor receptor (TNF-R) 1 recruit caspase-8 (and -10) which serves as the apical caspase in the death receptor pathway (Figure 1) [44].

Fas-induced apoptosis can follow two distinct pathways. In type I cells, sufficient caspase-8 activation occurs in order to process caspase-3 and caspase-7 which activate downstream caspases. In type II cells, low levels of caspase-8 activate Bid by cleaving it into a truncated form, tBid. Bid is a proapoptotic member of the Bcl-2 family. Upon activation it translocates from the cytoplasm to the outer membrane of the mitochondria where it provokes the release of cytochrome c (Cyt c). Cyt c release generates effector caspase activity that kills type II cells [47].

The death receptor pathway can be modulated by blocking different events of the cascade. Proteins such as inhibitor of apoptosis proteins (IAPs) inhibit the enzymatic activity of mature caspases by blocking substrate entry [47]. In contrast, FLICE-like Inhibitory Proteins (cFLIPs) have been thought to be effectively catalytically-inactive pro-caspase-8 molecules, although this seems to be true only for the short splice variant of cFLIP [44]. In type II cells Bcl-2 family members Bcl-2 and Bcl-X_L are able to confer protection from apoptosis by scavenging pro-apoptotic proteins [48].

Mitochondrial pathway

A variety of cellular stresses, including oxidative stress, γ-irradiation and removal of growth factors, can activate the mitochondrial apoptosis pathway. Little is still known about how these stimuli target various intracellular components, but at some point the signal leads to perforation of the mitochondrial outer membrane and the release of Cyt c into the cytosol. Cyt c then binds to apoptotic-protease-activating-factor-1 (Apaf-1) which triggers the formation of an apoptosome, a complex consisting of Cyt c, Apaf-1, ATP and procaspase-9 molecules. Processed caspase-9 acts as an apical caspase in the mitochondrial apoptosis pathway and, like caspase-8, effectively activates the effector caspases -3 and -7 which cleave the cellular substrates needed for controlled destruction of the cell. IAPs can inhibit caspase-9 but the mitochondrial proteins Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI) and Htr2A/Omi (5-hydroxytryptamine (serotonin) receptor 2A/Omi) are able to counteract IAP and allow progression of apoptosome-mediated apoptosis (Figure 1) [49, 50].

The Bcl-2 family of proteins plays an important role in the regulation of mitochondrial apoptosis [48]. The numerous members of the family can be subdivided into three classes: (1) anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L (Bcl-2-like survival factors), (2) BH3-only proteins, pro-apoptotic proteins named for their structure, such as Bid and (3) pro-apoptotic proteins, such as Bax (Bax-like death factors). The antiapoptotic members of the Bcl-2 family are associated with the mitochondrial outer membrane and serve to maintain mitochondrial integrity. The BH3-only proteins are normally inactive, but upon apoptotic stimuli activate some large pro-apoptotic members including Bax. As discussed above, Bid provides a link between the death receptor and mitochondrial apoptosis pathways [49]. The proapoptotic proteins associate with the outer mitochondrial membrane at apoptosis and disrupt its integrity [47, 48]. Bcl-2-like survival factors prevent membrane perforation and the release of the pro-apoptotic factors Cyt c, Smac/DIABLO and Htr2A/Omi [48].

Caspase-dependent apoptosis

Caspases are synthesized as inactive zymogens (the pro-form of the protein) that need to undergo proteolysis at activation. A commonly used, although most likely over-simplified, classification of caspases involved in apoptosis is to divide them into initiator and effector (or executioner) caspases. The initiator caspases have long prodomains that enable the recruitment of procaspases. These include caspases-2, -8, -9, -10, and -12 [51, 52]. They interact with various caspase-activating proteins and upon activation start cleaving effector caspases that amplify the apoptotic signal. The effector caspases, i.e. caspases-3, -6 and -7, lack large non-enzymatic domains. Caspase-3 has a central role in apoptosis, as the death-receptor and mitochondrial apoptotic pathways converge at the level of its activation. The effector caspases cleave substrates responsible for dismantling the cell [51]. One of the best characterized biochemical features of apoptotic cell death is the cleavage of nuclear DNA between the nucleosomes, resulting in multiples of 185 bp fragments [53, 54].

Caspase-independent apoptosis

Apoptotic insults do not necessarily need caspase activation for propagation. Bax, for example, is able to increase mitochondrial outer membrane permeability and induce apoptosis even when the activity of caspases is blocked [48, 55]. Mitochondria can release death effectors, such as apoptosis-inducing factor (AIF), that upon release translocate to the nucleus causing chromatin condensation and high-molecular-weight (50 kbp) DNA fragmentation [50, 55]. Htr2A/Omi also seems to be able to trigger caspase-independent cell death, although it may not be genuine apoptosis. Additionally, apoptotic stimuli may release mitochondrial endonuclease G which is normally responsible for repair of mitochondrial DNA, but may also act caspase-independently in apoptosis to degrade genomic DNA into high-molecular-weight and nucleosome-sized fragments [55].

Tumor suppressor p53

Cellular stresses, such as DNA damage produced by ϒ-irradiation, may lead to activation of the p53 tumor suppressor protein and thereby cause cell cycle arrest or apoptosis. In apoptosis, p53 may act through the death receptor pathway by promoting the expression of genes encoding death receptors. Alternatively, p53 may induce apoptosis through the mitochondrial pathway by inducing the expression of proapoptotic members of the Bcl-2 family, such as Bax and Bid, and inhibiting the activity of mitochondrial antiapoptotic molecules, such as Bcl-2 and Bcl-X_L. Additionally, p53 can transactivate components downstream of mitochondria, such as the genes encoding Apaf-1 and HtrA2/Omi. It can also inhibit the production of IAPs and thereby increase apoptosis. Disruption of p53 promotes inappropriate survival of damaged cells. It is the most commonly inactivated tumor suppressor gene in human cancers [56, 57].

Sphingolipids in apoptosis

Sphingolipids are essential structural components of cell membranes, but they also have an important role in cellular damage and death-response signaling. The sphingolipid ceramide is a lipid

second messenger that has over the past decade been shown to be involved in triggering apoptosis in several cell systems [58, 59]. Ceramide is mainly generated by cleavage of sphingomyelin (SM), a prominent phospholipid component of eukaryotic cell membranes (Figure 2). This cleavage occurs after rapid activation of sphingomyelinases. The best-studied sphingomyelinases for their roles in ceramide generation are the lysosomal acid pH optima sphingomyelinase (ASM) and neutral membrane-bound Mg^{2+}-independent sphingomyelinase (NSM). Both these enzymes can be activated by pro-apoptotic molecules [60], as well as extracellular stress stimuli, e.g. irradiation [58, 59] and growth factor deprivation [58, 59, 61, 62], which results in increased ceramide levels over a period of minutes to hours.

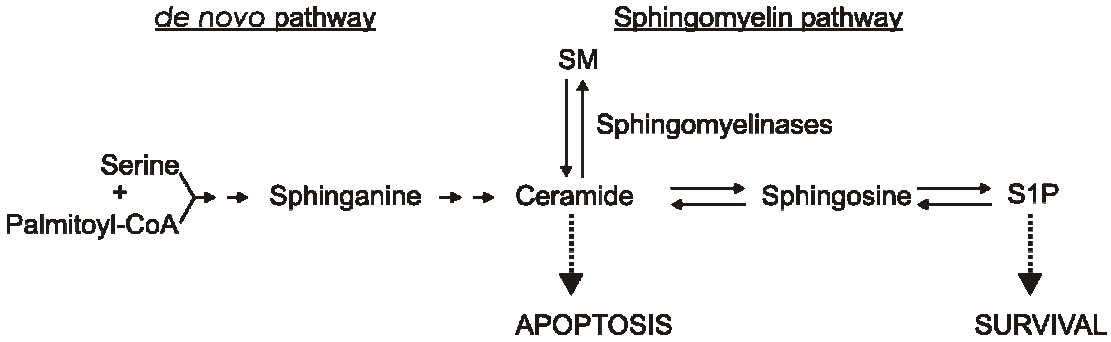

Figure 2. Metabolism of ceramide. Ceramide can be synthesized *de novo* from serine and palmitoyl-CoA (left) or by degradation of sphingomyelins via sphingomyelinases. Ceramide can be further metabolized into another supposedly proapoptotic lipid sphingosine. Phosphorylation of sphingosine yields S1P which opposes the cytotoxic actions of ceramide. Elevation of ceramide levels by the *de novo* pathway has been proposed to be a slow process leading to a prolonged rise in ceramide (hours to days), whereas sphingomyelinase action results in a rapid and more transient increase in ceramide (minutes to hours).

The importance of ASM action in response to various stress stimuli, e.g. irradiation, is demonstrated by the inability of many ASM-deficient cell types to undergo apoptosis. These cells include T- and B-lymphocytes and murine embryonic fibroblasts [60]. Their resistance to irradiation-induced apoptosis correlates with failure to generate ceramide in response to irradiation. However, re-constitution of ASM is able to restore both ceramide production and apoptosis. Interestingly, the apoptotic pathway chosen for cell execution is very dependent on the stimulus for apoptosis. The ASM deficient embryonic fibroblasts that are resistant to irradiation are sensitive to apoptosis caused by the chemotherapeutic drug staurosporine, which does not utilize ceramide-dependent apoptosis pathways [63]. Thus, it seems that the lack of ceramide, rather than of ASM, determines resistance to apoptosis.

The role of NSM in apoptosis is less defined, although it has been implicated, for example, in radiation responses [58]. Its activity is inhibited by glutathione and therefore conditions of oxidative stress, when glutathione is depleted, de-repress NSM [64]. Agents that decrease glutathione, such as TNFα, can therefore induce prolonged ceramide elevation through activation of NSM [64].

Ceramide can also be generated *de novo* from sphinganine or in a salvage process from sphingosine (Figure 2). Synthesis of ceramide through sphinganine requires coordinated action of several enzymes that orchestrate the process beginning from condensation of serine and palmitoyl-CoA. The salvage pathway re-utilizes sphingosine released from more complex sphingolipids. Drugs and irradiation may stimulate *de novo* ceramide synthesis, usually resulting in prolonged ceramide elevation [59].

Several cell death components seem to be involved in the mechanism by which ceramide triggers apoptosis. Ceramide may transcriptionally regulate cell death gene products, such as Fas-ligand (FasL) and TNFα [65]. Alternatively, ceramide may induce apoptosis directly without transcriptional activity by, for example, disrupting mitochondrial functions. It can inhibit the mitochondrial respiratory chain complex III, induce the generation of reactive oxygen species (ROS), and facilitate Bax induced Cyt c release [66]. It can also form large pores to the mitochondrial membranes which allow the outflow of Cyt c [67]. The release of Cyt c subsequently initiates downstream caspase activation. Indeed, activation of caspase-3 in response to ceramide has been found in several cell lines [68].

Once generated, in addition to accumulating in cells, ceramide can be converted back to SM or metabolized to another pro-apoptotic lipid, sphingosine, which can then be phosphorylated into S1P [68]. S1P seems to have a role intracellularly as a second messenger and extracellularly through specific receptors. The second messenger functions concern cellular proliferation and survival, but the intracellular targets have not yet been unequivocally identified [69]. S1P inhibits apoptosis induced, for example, by ceramide, TNFα, FasL as well as by other toxic agents [70]. The Cyt c release-dependent mitochondrial apoptosis pathway induced by various stimuli appears to be counteracted by S1P, and this has been suggested to occur upstream of caspase activation [71]. Importantly, S1P has been implicated as a sphingolipid rheostat that opposes the apoptosis-promoting effects of ceramide [70]. Thus, the balance between these two sphingolipid second messengers has been suggested to be an important factor in determining the survival or death of mammalian cells.

Extracellularly, S1P binds to a family of G-coupled receptors named $S1P_{1-5}$, originally known as endothelial differentiation gene (EDG) -1, 3, 5, 6 and 8 [72]. All five receptors bind to S1P with high affinity and after activation of G-proteins activate different intracellular signaling pathways, such as protein phosphorylation pathways and second messenger systems (cAMP and Ca^{2+}) [73]. Extracellular S1P regulates diverse cellular actions, such as cell migration, proliferation, cytoskeletal organization and morphogenesis. The mode of cell response seems to depend on cell type and relative expression of the receptor [72]. Whether S1P receptors are also involved in the regulation of apoptosis is currently unclear. S1P can move rapidly between membranes and, therefore, in addition to serving as an extracellular effector it may this way again become an intracellular mediator [69].

Physiological ovarian follicle apoptosis

In human fetuses, the number of oocytes reaches a maximum of seven million at 20 weeks of gestation, establishing a large reserve of primordial follicles [74-76]. Some of the newly formed

follicles start growing almost immediately, although most remain in the resting stage, and vast numbers undergo apoptotic cell death [24, 76]. The number of follicles drops to around one to two million at birth [24, 76]. Secondary and antral follicles can already be found in neonatal human ovaries. In infancy, when the circulating levels of gonadotropins remain low, follicles up to 2 to 6 mm can be observed. After the age of 6 years some follicles can exceed 6 mm as gonadotropin levels increase [24]. At the time of menarche 200 000 - 400 000 follicles, most of them primordial, reside in the ovaries [75, 77, 78]. Only up to 400 oocytes ovulate during the fertile period of life [24] and 1000 follicles remain in the ovaries at the onset of menopause, occurring at the median age of 51 years [79]. Thus, over 99% of follicles eventually undergo atresia [80]. Apoptosis has been demonstrated as a sign of atresia of follicles in various developmental stages [81, 82]. Oocyte apoptosis is most profound during fetal development [76]. However, during folliculogenesis apoptosis is mainly observed in the granulosa cells of growing follicles [83] and the process seems to be principally under hormonal control [24].

According to the two-cell two-gonadotropin theory, LH acts on the theca interna resulting in the production of aromatizable androgens, mostly androstenedione and some testosterone. Driven by FSH stimulus, these androgens are aromatized into estrogens by activation of the aromatase enzyme in the granulosa cells [24, 84]. Androgens also seem to be involved in human ovarian follicle atresia. Granulosa cells from preantral follicles smaller than 2 mm produce only low amounts of androgens and no estrogens, as they have only a low aromatizing capacity. From 2 mm upwards, as the follicles become selectable, they become more dependent on FSH and its absence leads to follicular atresia. Healthy and atretic 1 to 5 mm follicles have a low intrafollicular estrogen-androgen ratio. During the process of selection the follicle destined for ovulation starts producing large amounts of estradiol and the androgen concentration declines [24]. Thus, it has been postulated that androgens promote FSH action in the earliest stages of follicular growth, whereas in the pre-ovulatory stages androgens become inhibitory [85]. This has been supported by the finding that androgens stimulate early follicular growth, even at the stage of primordial follicles, and suppress granulosa cell apoptosis in the rhesus monkey ovary [86, 87].

Large atretic antral follicles (>6 mm) have an intrafollicular milieu that is dominated by androgens as opposed to estrogens in healthy follicles [88]. Androgen receptors (ARs) are down-regulated during pre-ovulatory development which serves to diminish the potentially harmful effects of androgens on large follicles [85]. The intracellular mechanisms underlying the actions of androgens in the follicles thus far remain unclear.

Pathological ovarian follicle apoptosis

As discussed earlier, the process of follicle atresia can be pathologically accelerated due to several reasons leading to premature ovarian failure (POF), i.e. cessation of ovarian function, before the age of 40. POF is observed in less than 1% of the general population. The underlying causes are iatrogenic, genetic, autoimmune or of environmental origin. Indications for the need of fertility preservation include gonadotoxic treatments for a disease and certain genetic conditions or diseases *per se* [89]. In cases where reduction in the human ovarian follicle reserve can be expected, measures to protect fertility should be taken.

Gonadotoxic treatments

POF is a common consequence of exposing the ovaries to chemotherapeutic drugs or irradiation used for carcer treatment, or of autoimmune diseases such as rheumatoid arthritis [89]. The risk of POF increases with age and the type of drug and/or dose of radiation delivered to the ovaries affects the outcome. Alkylating agents such as cyclophosphamide interact with DNA causing inaccurate base pairing and production of single- and double-stranded breaks. This inhibits DNA, RNA and protein synthesis, impairs follicular maturation and depletes the primordial follicle reserve. Irradiation has similar effects, leading to manifestation of the hallmarks of apoptosis and rapid pycnosis of the oocytes [89]. Animal studies have shown that follicles at different developmental stages have different sensitivities to irradiation, primordial follicles being the most sensitive [90]. Of clinical note, children conceived after chemotherapy and/or irradiation are not at increased risk of congenital anomalies [89].

Genetic factors

Genetic disorders also cause POF. Two fully functioning X-chromosomes appear necessary for the normal function of the ovaries. In Turner's syndrome, the most severe irreversible genetic cause of POF, one X-chromosome is completely or nearly completely missing. This usually leads to early and severe loss of ovarian follicles. Approximately 90% of 45,X0 girls have complete failure of pubertal development and no menarche [17]. However, adolescent girls with Turner's syndrome often possess follicles in their ovaries, which makes them suitable candidates for fertility preservation. It has been found that individuals with the lowest FSH levels have highest follicular densities. Additionally, the highest numbers of follicles can be found in girls with mosaic Turner's syndrome [15]. The mechanisms by which follicles undergo excessive atresia in the Turner ovaries is currently not known, although this might involve apoptosis. Postnatally, XO mice have approximately half as many oocytes as their XX sisters due to excess atresia at the late pachytene stage. It has been proposed that incomplete X chromosome pairing leads to the increased oocyte atresia [91].

Other X-chromosomal deletions and mosaicisms, including premutation carriers of fragile-X syndrome, have an increased risk of POF [17]. Genetic defects elsewhere in the genome (i.e. in autosomes) may also result in conditions that adversely affect fertility [17]. These include rare inherited disorders such as FSH receptor mutations [92].

Mechanisms of ovarian cell death

Many genetically manipulated knock-out (KO) mouse strains with deficient ovarian function have shed light on the mechanisms of ovarian follicle depletion. A non-functional Fas-FasL system in the mature ovary leads to defective follicle apoptosis [93], increased numbers of secondary follicles and irregular follicle development [94]. Knocking out the ataxia telangiectasia gene, believed to encode a protein principally involved in the cellular DNA damage response such as caused by irradiation,

results in massive germ cell loss during gametogenesis [95]. Additionally, mice with disrupted ASM [3] expression possess enlarged oocyte reserves due to attenuated fetal germ cell apoptosis.

The *Bcl-2* gene family has been shown to have an important role in physiological apoptosis in the ovaries [96-99]. Apoptosis of fetal germ cells in the mouse ovary is well correlated with increased Bax levels with no concomitant changes in Bcl-2 expression [100]. However, studies on young adult *Bcl-2* KO mice have revealed significantly reduced primordial follicle reserves [96, 101], but it is unclear whether this is due to excessive germ cell death during fetal development or increased apoptosis of follicles postnatally, or possibly both [102]. In contrast, Bax deficiency enlarges the oocyte reserve due to attenuated postnatal apoptosis of primordial and primary follicles [99]. *Bax* is an essential gene regulating both germ cell and granulosa cell demise [97, 98]. Accordingly, a shift towards Bax dominance over Bcl-2 in granulosa cells has been suggested to be a critical step in follicular atresia [103]. Other pro- and anti-apoptotic members of the Bcl-2 family may also function as key regulators of ovarian follicle survival, and it has been suggested that the fate of a follicle is ultimately determined by the end-result of the interaction between these proteins [102].

Mitochondria, the principle targets for Bcl-2-mediated regulation of apoptosis, are involved in granulosa cell apoptosis through the release of Cyt c and subsequent caspase activation [104]. Interestingly, caspase-3 has a central role in granulosa cell apoptosis [105] whereas caspase-2 is important for oocyte apoptosis [106, 107]. Caspase-3 deficiency does not affect oocyte death whereas granulosa cell apoptosis is aberrant [105]. Caspase-2 [107], as well as caspase-9 deficiencies [108], lead to enlarged oocyte reserve due to attenuated fetal germ cell apoptosis. Caspase-9 defective granulosa cells also show aberrant atresia [108].

Several components of the apoptotic machinery also lie behind ovarian follicle apoptosis induced by cytotoxic treatments. Oocytes from Bax [109], caspase-2 [107] and caspase-12 [110] KO mice are resistant to chemotherapy-induced death [109], whereas caspase-3 deficient females have impaired pathological granulosa cell apoptosis [105]. Targeted expression of the Bax antagonist *Bcl-2* in mouse female germ line also prevents chemotherapy-induced oocyte apoptosis [109, 111]. Ceramide may be a key stress sensor in oocyte apoptosis, as ASM-deficient oocytes are completely resistant to apoptosis induced by anti-cancer drug treatment [3]. Moreover, S1P, an inhibitor of ceramide-promoted apoptosis, completely protects the ovaries from radiation-induced damage, thereby preserving ovarian function and fertility in adult female mice that are exposed to irradiation [3]. This identifies the SM pathway as an important regulator of female germline death. Interestingly, p53-deficient oocytes are normally sensitive to chemotherapy [109, 111], indicating that p53 may not be involved in chemotherapy-induced apoptosis. It has been postulated that the pathway chosen for ovarian follicle apoptosis is dependent on the stimulus for apoptosis [102].

The theca regulates follicle survival via paracrine actions. However, little is known about the mechanism by which theca/stroma regulates the viability of ovarian follicles. Although granulosa cells are thought to be the first cell type to undergo apoptosis during atresia of maturing follicles, the theca layer ultimately undergoes apoptosis in order to allow rapid clearance of the non-viable follicle. Many antiapoptotic proteins have been localized to the theca, including Bcl-2, Bcl-X_L and some members of the IAP family, but little is known regarding their role in autocrine or paracrine regulation of cell survival in the ovary [112].

Preservation of female germ cells

Several strategies exist for preserving the human ovarian follicle reserve (Figure 3). However, the success of these methods varies. The lack of mature gametes brings an additional challenge to the fertility preservation of children.

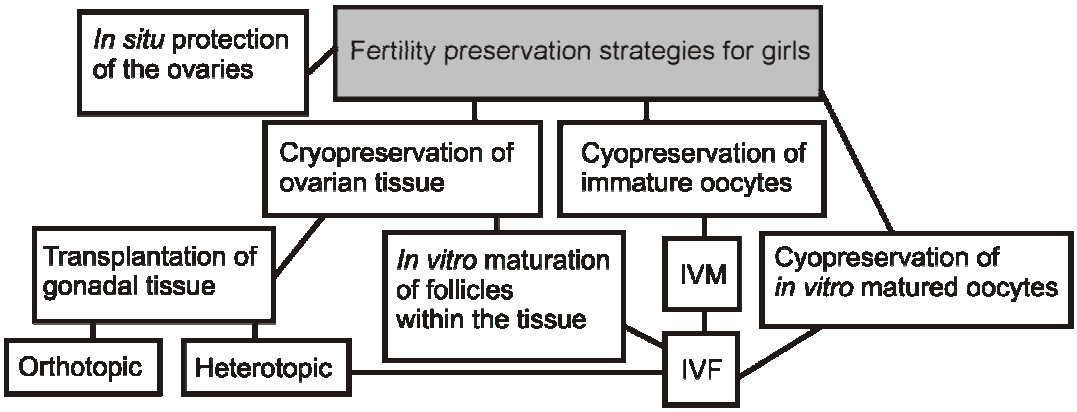

Figure 3. Alternatives for germ cell preservation in prepubertal girls. Prepubertal children do not possess mature germ cells. Therefore, it is vital to develop methods to mature follicles *in vitro* in cultures or *in vivo* within transplants. Another method would be to therapeutically prevent inappropriate ovarian follicle death during sterilizing treatments.

Transplantation of ovarian tissue

Cryopreservation of human ovarian tissue is currently feasible as over 70% of primordial follicles survive the freeze-thaw procedure, the follicles have normal morphological features after thawing and the tissue shows no signs of cell death [113-115]. Primordial follicles are the most common type of follicles within the tissue and they withstand low temperature banking rather well as they are small and metabolically relatively inactive and possess no zona pellucida, few cytoplasmic organelles and no spindle apparatus [40].

Ovarian grafts could potentially be able not only to restore fertility potential, but also provide a natural source of sex steroids. Ovarian tissue can be autografted orthotopically (onto the ovarian pedicle) or heterotopically (e.g. subcutaneously) [89]. Theoretically, natural conception is possible when orthotopic grafting is performed. Ovarian grafts have a relatively short life span, however, and longevity exceeding 3 years has not been confirmed. The frozen-thawed strips are regrafted into the patient without a vascular supply and may therefore suffer from ischemia-reperfusion injury. Substantial numbers of follicles have been reported to be lost during graft establishment [89]. In case only a few follicles remain in the tissue, a heterotopic site, such as a subcutaneous placement, would be favorable due to easy access of follicles for IVF. Other criteria for selecting a transplantation site include ample blood supply and no need for general anaesthesia or abdominal surgery [116].

Very recently three major steps have been taken in the field of transplantation. The first successful fertilization, pregnancy and birth of a healthy offspring was achieved in a primate (rhesus monkey) following egg collection from fresh transplanted ovarian tissue. The best follicular development was observed with abdominal subcutaneous grafts [117]. In another study, heterotopic transplantation of thawed human ovarian tissue beneath the skin of the abdomen resulted in a morphologically normal four cell embryo that was transferred to the patient's uterus [35]. However, no pregnancy ensued. Finally, a healthy baby was born in September 2004 from orhotopically autotransplanted ovarian cortical tissue that had been frozen for 6 years [34].

Xenotransplantation of human ovarian tissue into a host animal provides a means for cancer patients to avoid cancer cell transmission and relapse. Follicle maturation and corpus luteum formation have been demonstrated in human ovarian tissue xenografted into the subcutaneous space of immunodeficient mice [118]. The use of oocytes matured by this method however, are not acceptable at present, however, as animal pathogens may be transmitted to the offspring.

In vitro maturation of follicles

Another method to encourage follicle development and also to avoid the risk of cancer cell transmission is to culture human ovarian biopsies *in vitro*. However, the dilemma of how to obtain fully mature oocytes from the excised tissue containing mainly primordial follicles has not been solved. Full *in vitro* development of a primordial follicle into a mature ovulatory follicle and production of live offspring has been achieved only in mice, and it was not from cryopreserved tissue. A two-step culture protocol was employed, in which organ cultures of newborn ovaries first produced preantral follicles that were then isolated and cultured further as oocyte-granulosa cell complexes [1, 2]. Interestingly, the total time for complete oocyte maturation in this *in vitro* protocol was comparable to that observed *in vivo*.

Human oocytes may need a similar kind of *in vitro* maturation procedure, starting with culturing of ovarian tissue followed by isolation and maturation of immature follicles. However, data obtained from mice cannot be applied directly to humans. The human ovary has a more dense structure, ovulates only one oocyte per cycle, oocyte density decreases markedly with age and most importantly, the time required for intensive growth and complete follicle maturation is approximately 120 days [119] as compared to 16 days in mice [120]. This may, however, be much shorter *in vitro* than *in vivo*, as in culture follicle growth seems to be highly accelerated. Many human ovarian follicles in tissue slices initiate growth within a week [30, 31, 33, 121] and the follicles regularly reach a secondary stage after 2 weeks in both fresh and frozen-thawed organ cultures [30, 33]. Thereafter the development seems to halt, although antral follicles can occasionally be seen within 3-4 weeks [30]. Although growth and development occur in the tissue slices, substantial progressive atresia is also present [30, 31, 33, 121].

Enzymatic isolation of human primordial follicles does not seem to improve their viability, as they survive only 24 h in culture [122]. Enzymatically isolated preantral stage follicles have been cultured for up to 5 days [123] and mechanically isolated preantral follicles for up to 4 weeks [124]. The mechanically isolated preantral follicles grew to early antral stages while the small antral follicles enlarged in culture. It has been assumed that mechanical isolation is more advantageous because enzymatic isolation results in severe damage or loss of the theca layer which is essential for

normal growth and production of estrogen precursors [124]. The use of preantral and antral follicles, however, is hindered by the rarity of these follicles in ovarian biopsies, and therefore maturation of the abundant primordial follicle reserve would be more practical.

Another alternative would be to encourage the primordial follicles to initiate growth within tissue cultures (7-9 days) and then partially isolate the follicles. However, partially isolated tissue often extrudes oocytes from secondary follicles, possibly due to suboptimal culture conditions [31], and this approach could also face the problem of having insufficient theca. Partial isolation is inferior to non-isolated tissue in regards to follicle growth and survival, and therefore it can be assumed that the optimal method for human ovarian follicles is to culture them within intact tissue slices where integrity of all cell types is preserved [31].

Recently, attention has focused on the search for factors that support human follicle growth *in vitro*. Culture within extracellular matrix [30] and supplementation of the culture medium with FSH [33] was shown to improve the survival and growth of human ovarian follicles within tissue slices. FSH decreases the percentage of atretic follicles and increases follicle diameter during a 15-day culture period. In addition, insulin increases the percentage of healthy follicles, and insulin, insulin-like growth factor (IGF) -I and IGF-II increase the percentage primary stage follicles after a 2-week culture [125]. Growth and differentiation factor-9 (GDF-9) improves the growth and survival of primordial, primary and secondary follicles, so that the majority of follicles reach secondary stage after culture for 2 weeks [121]. Cyclic guanosine monophosphate (cGMP) enhances follicular growth to the secondary stage and improves follicle viability in 1- and 2-week cultures [126]. Cyclic adenosine monophosphate (cAMP) also promotes folliculogenesis in 2-week cultures and improves follicle survival in 3-week cultures [127].

Maturation of cryopreserved oocytes

The prepubertal human ovary contains constantly developing follicles that reach antral stages before attrition. When antral follicles are found in excised human ovarian tissue, it is possible to aspirate the germinal vesicle-stage oocytes from the follicles [128]. These oocytes can either be frozen immediately or matured *in vitro* and cryopreserved thereafter. Immature, *in vitro* matured and mature oocytes survive freezing [129].

Mature oocytes have been thought to be more prone to cryoinjury than germinal vesicle oocytes as they have a larger cell size and a meiotic spindle that is easily damaged by variations in temperature, resulting in chromosomal instability [89]. At present, although high survival rates have been reported after freeze-thawing mature oocytes [130], successful pregnancies are rarely achieved. However, pooled data from the literature has not shown any advantage of immature oocyte freezing in terms of oocyte survival, fertilization rates of *in vitro* matured oocytes and developmental competence of the resulting embryos [129]. Nevertheless, a birth generated from a cryostored immature germinal vesicle stage oocyte [131] gives hope for the future use of frozen immature follicles.

In situ protection of ovaries

Another method for fertility preservation would be to protect the ovaries *in situ* from external insults leading to cell death. Ovarian transposition before pelvic radiotherapy can reduce the dose to the ovaries to approximately 5%-10% of that in untransposed ovaries, although ovarian transposition itself may increase the risk of POF even without radiation therapy [89]. Studies on animals have indicated that suppression of the pituitary-gonadal axis may provide some protection against ovarian follicle depletion. Postpubertal ovaries have been rendered inactive by administration of GnRH analogs [132, 133] or contraceptives [134], but there is no conclusive evidence of significant protection, which is in accordance with the fact that prepubertal gonads are indeed susceptible to gonadotoxic agents [9, 10].

Since follicle death is known to occur by apoptosis, recent studies in mice have explored whether this process can be switched off by pharmacological manipulation of the apoptosis pathway. S1P given locally into the ovarian bursa prior to irradiation completely blocks irradiation-induced primordial follicle depletion [3]. It has also been confirmed that no discernible genomic damage is propagated to the offspring of the protected mice [135]. This potential treatment is currently under clinical development.

Physiological male germ cell apoptosis

In human testes adult type spermatogonia first appear between birth and the age of 6 months [136], whereas in mice spermatogonia are present by day 8-post partum [137]. In addition to stem cell spermatogonia, the human prepubertal testis contains differentiating spermatogonia. Occasional spermatocytes are also encountered, but they appear to degenerate or progress into abnormal spermatids, which also face degeneration [25, 26]. The prepubertal testis therefore seems to be "quietly active" rather than dormant [138]. At the beginning of puberty primary spermatocytes appear shortly after establishment of spermatogenesis and sperm production reaches normal adult levels by 14 or 15 years of age [25, 26]. In the mouse, increasing numbers of secondary spermatocytes and haploid spermatids appear between days 18 and 20, thereby denoting active spermatogenesis [137]. Mature spermatozoa arise at puberty, at approximately day 30-post partum [139].

In the mouse, the first wave of spermatogenesis is accompanied by massive germ cell apoptosis around the age of 3 weeks [140, 141]. Cell death is not restricted to spermatogonia but may in fact affect mainly spermatocytes [140, 142]. This apoptosis is mediated by caspase-3 [142] and is most likely required to maintain the correct number of maturing germ cells per Sertoli cell [141]. Bax, the pro-apoptotic member of the Bcl-2 family, seems to be essential for adequate germ cell death during the first wave of spermatogenesis. At this time Bax is abundantly expressed in the testis and localized to apoptotic germ cells [140]. When the early apoptotic wave is disturbed, as in Bax KO mice [143] or mice expressing high levels of the anti-apoptotic Bcl-X_L or Bcl-2 proteins [140, 144], spermatogenesis is abnormal and sterility ensues.

The incidence of adult male germ cell apoptosis also remains high, as the majority of potential spermatozoa degenerate in the testes [46, 141, 145]. Substantial death of germ cells is a

regular feature of normal spermatogenesis [46, 146]. This can be explained at least in part by selective elimination of damaged or defective germ cells [46, 141]. For example, spermatocytes that carry unpaired chromosome segments seem to face apoptosis [141]. Interestingly, while in humans spermatogonia, spermatocytes and spermatids undergo spontaneous apoptosis, in the mouse spontaneous apoptosis is most commonly observed in spermatocytes, but less frequently in spermatogonia and seldom in spermatids [147, 148]. However, the exact incidence of adult male germ cell apoptosis is difficult to estimate as not all dying germ cells display the classic features of apoptosis. Degenerating spermatogonia and round spermatids most likely die by apoptosis since they exhibit many biochemical and morphological features of apoptosis. The method of cell death of spermatocytes and elongated spermatids is less unequivocal since they do not exhibit the characteristic nuclear changes typical of apoptosis. This may be due to their unusual morphology and configuration of DNA [141].

Pathological male germ cell apoptosis

Inappropriate germ cell apoptosis may occur in testicular germ cells in response to cell injury or stress, such as irradiation, alterations in hormonal support or toxin exposure [149-153]. As with females, childhood cancer in males is one of the clearest indications of the potential need of fertility preservation. Boys having experienced cancer in childhood often manifest sub- or infertility as adults [11].

Gonadotoxic treatments

Cancer therapy often damages the germinal epithelium, leading to oligozoospermia (reduced numbers of spermatozoa) or azoospermia (no spermatozoa). The cell populations most sensitive to cancer treatments are spermatogonia and primary spermatocytes. The impact of chemotherapy depends on the type and dosage of the drugs received. Some drugs, such as alkylating agents, can cause significant damage to the germ cells. Although some studies have indicated that younger males would be less vulnerable to the toxic effects of chemotherapy, recent studies have not supported this claim. Impaired sperm production is a common consequence of irradiation as well. This method of treatment also causes depletion of the germ cells in prepubertal boys. It seems that total body irradiation is especially harmful for the recovery of fertility [11].

Although both chemo- and radiotherapy easily harm germ cells, Leydig cell function is often preserved. Chemotherapy-induced Leydig cell failure resulting in androgen insufficiency is quite rare. Leydig cell function is preserved up to a 12 Gy radiation dose. Thus, boys may progress through puberty despite depletion of the germinal epithelium [11].

Normal sperm production and fertility may in some cases follow cancer treatments after a non-specific delay. One year delays are common but the delay may be 3 to 4 years or even longer. In contrast, some patients may have adequate sperm counts and quality immediately after cancer treatments which subsequently worsen over time. In such patients the recovery is hard to predict and some may become permanently sterile [154]. Much concern has been raised relating to the

potential risk of fetal malformations in the offspring of treated patients. However, several studies have provided reassuring results showing no such long-term adverse effects [11].

Genetic factors

Genetic factors that adversely affect male fertility are manifold. Klinefelter's syndrome is among the most common chromosomal disorders associated with male infertility. In most cases the syndrome is characterized by 47,XXY, although other variants include 47,XXY/46,XY mosaicism, 48,XXXY, 48,XXYY and 49,XXXXY. In general, patients with mosaicism are less affected [18]. In early adolescence as many as 50% of the non-mosaic patients may have germ cells in their testes, and depletion of the germ cells accelerates at the onset of puberty [16]. In adulthood spermatogenesis is very rare in these patients. Thus, cryopreservation of testicular biopsies or semen samples may preserve future fertility of boys and young men for whom the syndrome is identified before they present with infertility [18].

Although the mechanism behind this germ cell depletion is unclear, it may be related to apoptosis. Normally spermatocytes that are unable to complete meiosis appear to face deletion by apoptosis [141]. However, different opinions exist whether germ cells from 47,XXY males can proceed through mitosis and meiosis to generate hypohaploid gametes. Spermatozoa of Klinefelter men have an increased frequency of chromosomal abnormalities. The genetic imbalance in the spermatozoa has been explained by two different hypotheses. First, 47,XXY spermatogonia may undergo meiosis but produce hyperploid spermatozoa. Second, rare patches of normal XY germ cells in XXY men produce spermatozoa, but these germ cells are susceptible to meiotic abnormalities due to a compromised testicular environment. However, most infants born after ICSI of ejaculated or testicular spermatozoa have a normal karyotype [18].

Mechanisms of testicular germ cell death

Several components of apoptotic cascades seem to be involved in physiological male germ cell death. Human germ cells carry Fas receptors while Sertoli cells possess both Fas receptor and FasL [155]. It has been suggested that Sertoli cells regulate germ cell numbers by releasing FasL, inducing apoptosis by an autocrine mechanism targeted against Sertoli cells or a paracrine mechanism targeted against germ cells [156]. Bcl-2 family members also play a fundamental role. Bax KO mice possess excessive numbers of spermatogonia and spermatocytes, probably resulting from the impaired removal of these cells during the first wave of apoptosis [143, 157]. Although supranumery germ cells are later deleted, most likely by Bax-independent apoptosis, the mice never achieve normal spermatogenesis and are infertile. The role of Bax in normal adult male mice is unclear, as it is expressed at low levels [140] and only in spermatogonia [158]. However, it is most likely involved in pathological germ cell apoptosis since it is upregulated in a human germ cell line accompanying cytotoxic drug-induced apoptosis [159].

Mice lacking the anti-apoptotic Bcl-w are also infertile [160, 161]. Their first wave of spermatogenesis is largely unaffected but the seminiferous tubules of adult males are disorganized, contain numerous apoptotic cells and produce no mature sperm [160, 161]. Accordingly,

overexpression of Bcl-2 or Bcl-X_L leads to disorganized seminiferous epithelia [140]. Bcl-X_L potentially promotes the survival of germ cells during embryogenesis and during the first wave of spermatogenesis, leading to a pathology in young mice similar to that of Bax KOs [140]. The phenotype produced by *Bcl-2* overexpression is essentially similar [144]. Many other Bcl-2 family members are also expressed in the testis but their roles in germ cell apoptosis have not been unequivocally clarified.

Apoptosis may be initiated by different stimuli in different types of testicular cells, which may utilize a variety of apoptotic pathways. Irradiation, for example, induces mouse germ cell apoptosis *in vivo* mainly among the differentiating spermatogonia as opposed to natural apoptosis that occurs mainly in spermatocytes [153]. Irradiation seems to upregulate Bcl-2 and Bcl- X_L, whereas Bax expression remains unchanged [162]. Heat-induced germ cell death is preceded by redistribution of Bax and further accompanied by Cyt c release, and activation of caspases-9, -3, -6 and -7 [163].

Detailed investigations of human germ cell apoptosis have been performed *in vitro* with a tissue culture model in which the physiological interactions of germ cells and Sertoli cells are maintained [164]. Dying germ cells, mainly spermatocytes and spermatids, are evident after only a few hours in culture [164]. The death receptor ligands FasL and TNFα along with caspases are involved in this *in vitro*-induced germ cell death. While FasL is pro-apoptotic, TNFα seems antiapoptotic through regulation of the level of FasL [165, 166]. Nuclear factor-κB (NF-κB), a transcription factor considered to be a major regulator in immune and stress responses, exerts pro-apoptotic effects on germ cells [167], and mitochondria seem to be intimately involved in propagation of stress-induced apoptosis [168, 169]. Additionally, the findings that ceramide levels in human seminiferous tubules increase rapidly *in vitro* before caspase-3 activation or DNA laddering, and that S1P suppresses this apoptosis [170], point to the involvement of ceramide in male germ cell apoptosis. Interestingly, hormone withdrawal *in vitro* seems to induce caspase activation in Sertoli cells, whereas acute apoptosis of germ cells appears caspase-independent [171]. Gonadotropins and testosterone are essential regulators of germ cell fate, the removal of which induces apoptosis [141, 164]. In addition to testosterone, estradiol appears to be a potent survival factor in *in vitro*-induced apoptosis [172]. The exact mechanisms by which gonadotropins, androgens and estradiol mediate their pro-survival effects on the germ cells remain undetermined.

p53 has been implicated as a guardian of the genome in the testis. In the mouse, p53 is normally expressed in spermatocytes but after irradiation-induced injury it is also detected in spermatogonia and appears to be involved in spermatogonial apoptosis [173, 174]. p53 KO mice have less spontaneous germ cell apoptosis, resulting in increased numbers of defective germ cells and reduced fertility [175]. p53 null mice also exhibit decreased apoptosis of differentiating spermatogonia after exposure to irradiation [176]. Overexpression of p53 results in excessive apoptosis of spermatids and reduced sperm production [177].

Preservation of male germ cells

A number of methods are being investigated for fertility preservation of prepubertal boys (Figure 4). However, at present all procedures remain experimental and no provisions exist.

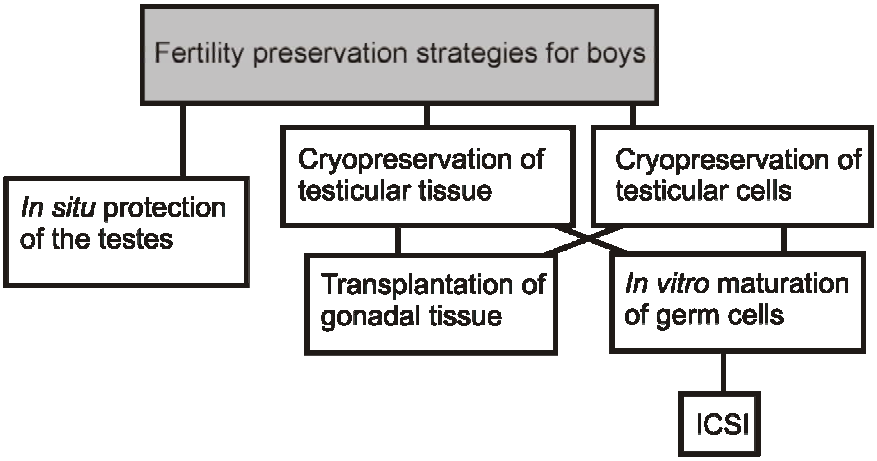

Figure 4. Alternatives for germ cell preservation in prepubertal boys. Testicular tissue cryopreservation as well as cryopreservation of testicular cell suspensions are feasible. However, no reports currently exist of successful *in situ* protection of the testes or of testicular tissue/cell transplantation, or of *in vitro* maturation of germ cells.

Transplantation of germ cells

Sperm banking is an effective way to preserve male fertility [23] but this technique is not available to prepubertal boys due to the absence of haploid gametes. Therefore, it is vital to develop a method that could support early germ cell differentiation to the stage of maturity that allows ICSI. Testicular tissue can be cryopreserved from infertile men either as cell suspensions [178, 179] or pieces of tissue [180, 181], and these techniques could also be used for prepubertal boys.

An attractive approach to male fertility preservation is germ cell transplantation. The objective is to remove germ cells from the testis before radio/chemotherapy and re-introduce them into the male gonad after the patient is cured. Spermatogenesis has been reconstituted in mice using cryopreserved spermatogonia [182, 183]. In primates (monkey and man), testicular cell suspensions have been transplanted successfully through injections into the rete testis. In the monkey, transplanted spermatogonia could be detected 4 weeks after autologous transfer [184]. Malignant cells could be removed from the cell suspension using a magnetic cell separation technique that utilizes c-kit antibodies for identification of spermatogonia [185]. This is necessary as only a few cancer cells may cause a cancer relapse [38]. Attempts to re-inject frozen-thawed testicular cells have also been made in men [186]. This application however, has not been reported to be successful so far.

By xenografting, rat spermatogonial germ cells transplanted into mouse testes are able to produce viable germ cells [187], but although primate (baboon) spermatogonial stem cells are able to repopulate rodent (mouse) testes, the stem cells do not differentiate in the rodent seminiferous epithelium [188].

Transplantation of testicular tissue

In mice, live offspring have been born following intratesticular transplantation of cryopreserved immature testicular pieces and subsequent ICSI [189]. Tissue transplantation may be more

advantageous than spermatogonial cell transplantation because it is a relatively simple procedure (no enzymatic digestion or ultrasound guidance of microinjection) and a small number of stem cells within the tissue is sufficient to recolonize seminiferous tubules. Additionally, as opposed to spermatogonial stem cell transplantation, with pieces of tissue the co-transplanted Sertoli cells may alleviate any hormonal imbalances caused by gonadotoxic treatments. Xenogeneic transplantation may also allow investigation of whether the biopsy is contaminated by malignant cells [189]. In a very recent study encouraging results were obtained by grafting testis tissue from immature rhesus monkeys into castrated immunodeficient mice. Acceleration of testicular maturation was observed and fertilization-competent sperm were produced. Accelerated testicular maturation was achieved without hormonal supplementation of the host mice, indicating functional hormonal crosstalk between cells of the two species [190].

In vitro maturation of male germ cells

Recently, an *in vitro* culture system was developed which supports the survival of spermatogonia from prepubertal boys for at least 3 weeks [191]. In another study, co-cultures of human Sertoli cells, spermatogonia and spermatocytes resulted in some degree of spermatogonial proliferation, resumption of spermatocyte meiosis and differentiation of round spermatids to elongated spermatids. The *in vitro* differentiated late spermatids with normal appearance nevertheless displayed low fertilization potential and high frequency of abnormal embryos [39]. However, round and elongated uncultured spermatids [192, 193] have previously resulted in viable embryos and live human births. Normal oocyte-activating competence has also been demonstrated even with secondary spermatocytes [194]. Complete spermatogenesis *in vitro* nevertheless seems a remote possibility as it has not yet been successful in any animal species [27]. The freeze-thaw procedure also brings an additional challenge to the method.

In situ protection of testes

Reversal of radiation and chemotherapeutic agent-induced infertility has been achieved in experimental animals by pretreatment with hormones that suppress spermatogenesis prior to irradiation. The original aim of hormonal treatment was to disrupt the pituitary-gonadal axis, rendering the testis quiescent (equivalent to an immature state) and thereby more resistant to cytotoxic insults. In fact, hormones that suppress LH, intratesticular testosterone production and completion of spermatogenesis seem to confer germ cell protection [195]. In the rat, damage caused by procarbazine has been reduced, for example, by testosterone alone or by testosterone combined with estradiol or with a progestin, by clomiphene and by GnRH agonists [195]. The effects of irradiation have been suppressed by pretreatment with testosterone [196], testosterone combined with estradiol [197, 198] and other hormonal combinations [199]. Pretreatment with GnRH antagonists and possibly with GnRH agonists also protect the rat testes against x-radiation [200].

The initial hypothesis of resistance of the immature testis to cytotoxic exposure has been challenged by the observation that in mice complete gonadotrophin deficiency does not confer any cytoprotection to the testis [201]. Moreover, the seemingly quiescent primate adolescent testis is susceptible to damage caused by cancer treatments [138]. It seems that the mechanism of hormonal

protection does not involve protection of stem cell spermatogonia from death but rather enhancement of the surviving spermatogonia to maintain differentiation and continue spermatogenesis [195]. It has been suggested that a temporarily reduced level of intratesticular testosterone stimulates the recovery of spermatogenesis [202]. Thus, suppressing gonadal function with GnRH analogs or with testosterone after irradiation or chemotherapy may result in more successful preservation of fertility, as indicated by many animal studies [195]. Interestingly, hormone receptors have not conclusively been shown to be expressed by spermatogonia, so that after irradiation it may be the Sertoli cells that become impaired and therefore unable to support spermatogonial differentiation. Transient changes in hormone levels may help the recovery of Sertoli cells [203]. However, enhancement of recovery of spermatogenesis may be effective only when the testicular insult is less severe so that many spermatogonia are preserved [202].

Despite several attempts, hormonal protection of the seminiferous epithelium from cytotoxic treatments in men have failed and no convincing data on subsequent fertility preservation has been presented [204, 205]. Interspecies differences may well exist in hormonal protection of the testes. Attempts to protect mouse spermatogenesis by antiandrogens, GnRH agonists and GnRH antagonists have been unsuccessful [206, 207]. GnRH antagonist treatment is also unable to provide gonadal protection in a non-human primate model [208]. In the rat model, many stem cells survive cytotoxic treatments but are unable to differentiate beyond the stage of spermatogonia. In humans only a few or no spermatogonia may survive gonadotoxic treatments and differentiation is blocked at the spermatocyte stage. In addition, rodent spermatogenesis proceeds to the spermatocyte and early spermatid stage in the absence of androgens and FSH, whereas in humans it is blocked at the spermatogonial stage [209].

Another possibility to reduce rodent germ cell damage caused by irradiation is non-hormonal protection of the gonads. For example, hypoxia [210], vitamin C [211] and an oriental traditional medicine *Panax Ginseng* [212] have been claimed to render spermatogonial stem cells more resistant to irradiation than untreated spermatogonia. The applicability of the above components to humans is not known.

In vitro studies of human testicular tissue have shown that the testis is responsive to pharmacological manipulation of apoptosis-related pathways [167, 168, 170]. The positive results obtained for the protection of oocytes with S1P have raised hopes that pharmacological inhibition of inappropriate apoptosis may become an option for future male fertility preservation *in vivo*.

AIMS OF THE STUDY

The present series of studies aimed at developing methods for the culture of human ovarian cortical tissue *in vitro* and finding a technique to pharmacologically protect testes *in vivo* from irradiation-induced germ cell death.

In particular, the following issues were addressed:

1. Investigation of the mechanism by which ovarian tissue dies under culture conditions and whether this cell death can be manipulated.

2. Whether the death of cultured ovarian tissue can be suppressed by the endogenous steroid hormones testosterone, dihydrotestosterone and 17β-estradiol.

3. Involvement of the ASM pathway in testicular and germ cell development and physiological apoptosis and in apoptosis induced *in vivo* by irradiation and *in vitro* by culture.

4. The possibility to prevent irradiation-induced male germ cell death *in vivo* by interfering with apoptotic pathways by using S1P.

MATERIALS AND METHODS

Details of the reagents used in these studies are referred in the original publications (I-IV).

Patients (I-II)

For ovarian tissue cultures, ovarian biopsies were obtained from 71 women, aged 18-38 years, who underwent gynecological operations for benign conditions. They had no diagnosis of ovarian dysfunction and donated tissue with informed consent. The operations were performed at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, and the Helsinki City Maternity Hospital (Helsinki, Finland). For cell cultures, granulosa cells were obtained from 8 women, aged 27-36. They were recruited at the Infertility Clinic of The Family Federation of Finland and were participants of an *in vitro* maturation (IVM) program. The women were regularly menstruating and were undergoing oocyte retrieval, maturation and *in vitro* fertilization because of tubal obstruction or male factor infertility. The women did not undergo ovary stimulation and gave informed consent before oocyte retrieval.

Animals (III-IV)

For studies on how mouse testes respond to irradiation and on the ability of S1P to protect the testes from irradiation, 8 to 10-week-old young adult wild-type C57BL/6 male mice were obtained from the University of Helsinki experimental animal facilities. For studies on the effects of ASM deficiency on spermatogenesis and germ cell apoptosis, male mice with a mixed 129/SV-C57BL/6 genetic background were obtained from the Vincent Center for reproductive Biology, Boston, Massachusetts. A polymerase chain reaction genotyping method was used to choose wild type (WT) and acid sphingomyelinase homozygous-null (ASMKO) littermates for the experiments. The mice analyzed were 7 days, 21 days, 8 weeks and 20 weeks of age. 8 to 10-week old young adult mice were used for the *in vivo* and *in vitro* apoptosis induction experiments.

Tissue and cell cultures

Long-term ovarian tissue culture (I)

The ovarian biopsies were cut into slices and the tissue was cultured for 1 or 3 weeks (long-term cultures) in inserts precoated with extracellular matrix and placed in 24-well plates containing culture medium. The tissue culture medium contained Earle's balanced salt solution, sodium pyruvate and antibiotics, supplemented with human serum, FSH and insulin. The medium was changed every other day. After incubation, the tissue was fixed in Histochoise tissue fixative.

Short-term ovarian tissue culture (I-II)

For short-term ovarian tissue cultures, the tissue was first handled as described above. The culture medium was supplemented with human serum albumin, gentamicin, FSH and insulin. The 0 h control samples were immediately snap-frozen in liquid nitrogen for Southern blot analysis, fixed in formalin for immunohistochemical analysis or Bouin's solution for morphological analysis. The slices of tissue were transferred to 24-well culture plates containing culture medium and cultured for 8 h, 24 h, or 48 h at 37°C in a humified atmosphere containing 5% CO_2. After culture, the tissue was either snap-frozen in liquid nitrogen or fixed in formalin or Bouin's solution.

The effects of an antioxidant (NAC) and some common natural steroids (testosterone, DHT and 17β-estradiol) on ovarian tissue viability were studied by adding them to the culture media of the 24-hour cultures. An AR antagonist bicalutamide (Casodex) was used simultaneously with DHT. The steroids were dissolved in ethanol. All cultures, including the controls, contained equal amounts of ethanol.

Granulosa cell cultures (II)

After oocyte retrieval for IVM and subsequent IVF, excess immature granulosa cells were collected from the aspiration fluid and separated from red blood cells as previously described [213]. The granulosa cells were collected and washed, resuspended in serum-free culture medium (the same as for the short-term tissue culture) and plated at a density of approximately 50 000 cells/ml. The effects of DHT on the granulosa cells were studied by culturing the cells with or without DHT for 24 h. The cells were then snap-frozen in liquid nitrogen for Southern blot analysis of DNA fragmentation.

For immunohistochemistry the granulosa cells were enzymatically dispersed with hyaluronidase and separated from red blood cells. The cells were cultured for 24 h on cell culture slides at a density of approximately 50 000 cells/ml in M199 medium containing inactivated fetal calf serum (FCS), penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. After culture the slides were rinsed and fixed in paraformaldehyde.

Testicular tissue cultures (III)

Apoptosis of mouse testicular germ cells was induced *in vitro* by incubating segments of seminiferous tubules under serum-free conditions according to the method described for human testicular tissue [164-169, 172, 214, 215], with some modifications. Testes were decapsulated in phosphate-buffered saline on a petri dish and the seminiferous tubules were microdissected in tissue culture medium supplemented with bovine serum albumin and gentamicin. The segments of seminiferous tubules were incubated at 34°C under serum-free conditions in a humified atmosphere containing 5% CO_2. The rate of apoptosis induction was initially shown by culturing mouse germ cells in serum-free conditions. Following the culture, the tissue was snap-frozen in liquid nitrogen and stored in -80°C for Southern blot analysis of fragmented DNA. We then chose two time points, 24 h and 48 h, for further *in vitro* experiments.

In order to compare the ability of WT and ASMKO mouse germ cells to die apoptotically, segments of seminiferous tubules from both groups were cultured for 0 h (freshly snap-frozen), 24 h or 48 h in serum- and hormone-free culture conditions. After culture the tubules were frozen in liquid nitrogen for Southern blot analyses and for analyses of ceramide and SM levels and squashed stage-specifically on microscope slides under cover slips for ISEL analyses of apoptotic cells.

In vivo treatments

Irradiation (III-IV)

Mice were anesthetized 1-2 h before irradiation after which they received a dose of 0, 0.1, 0.5, 1.0, or 2.0 Gy total-body irradiation at the Department of Oncology, Helsinki University Hospital, Helsinki, Finland. Irradiation was performed using a 6-MV photon beam and a dose rate of 2 Gy/min. The mice were placed in a plastic box and irradiated by means of a single posterior field covering the whole box and a 2 cm margin to achieve maximum uniformity of dose distribution. Sixteen hours after irradiation the testes were weighed, one testis was snap-frozen for measurements of ceramide and sphingosine levels, and the other was used for stage-specific squash preparations for ISEL analysis of DNA fragmentation. Small tissue samples were also fixed in glutaraldehyde for electron microscopic (EM) studies. After 21 days testes from another group of mice were weighed, samples taken for EM, and stage-specific DNA flow cytometric analyses performed.

S1P experiments (IV)

A control group of animals received an intratesticular injection of vehicle in phosphate-buffered saline (PET-PBS) into the right testis while the left one remained as an untreated control. The S1P-treated group of mice received injections of 50 μM S1P in PET-PBS into the right testis and 200 μM S1P in PET-PBS into the left testis. At the 16-h time point, the effects of S1P on irradiation-induced (0.5 Gy) rapid germ cell demise were studied from stage-specific squash preparations by ISEL analysis of DNA fragmentation or by EM (0.5 and 1.0 Gy) from tissue samples fixed in glutaraldehyde. At the 21-day time point, after stage-specific preparation of seminiferous tubules, the long-term effects of S1P on irradiation-induced (0.5 Gy) apoptosis were studied by DNA flow cytometry.

Laboratory analyses

Histology (I-II)

Tissue from short-term cultures was fixed in formalin or Bouin's solution, dehydrated and embedded in paraffin blocks. The blocks were cut into serial sections, mounted on slides and stained with hematoxylin and eosin for histological evaluation, or alternatively stained with the

ISEL method. The long-term cultures fixed in Histochoise were embedded in paraffin, cut into serial sections, and stained with hematoxylin-eosin in order to analyze the viability of the follicles.

Electron microscopy (I, III-IV)

Tissue was fixed in glutaraldehyde in phosphate buffer, dehydrated, and embedded in epoxy resin. The samples were sectioned and stained with uranyl acetate and lead citrate. Observations were made with a transmission electron microscope.

Southern blot analysis of DNA fragmentation (I-III)

Snap-frozen tissue was stored at -80^{o}C until DNA isolation. DNA was extracted using the Apoptotic DNA Ladder Kit according to the manufacturer's instructions, with a few modifications. Briefly, the tissue was homogenized and incubated at room temperature in binding/lysis buffer. After quantitation, DNA samples were 3'end-labeled, subjected to electrophoresis on agarose gels and blotted onto a nylon membrane, as previously described [164]. The 3'end-labeled DNA fragments on the nylon membranes were detected with anti-digoxigenin antibody and the chemiluminescence reaction, as described [164]. X-ray films were exposed to the luminescent membranes and scanned. The optical density readings from the films were transformed to pixels and the digital image analysed.

Nonradioactive in situ end labeling (ISEL) of apoptotic DNA (I-IV)

Formalin-fixed and paraffin-embedded ovarian tissue sections were deparaffinized, rehydrated, and washed in distilled water. Alternatively, isolated 1-mm segments of seminiferous tubules were transferred to microscope slides and the samples were squashed by placing a coverslip on top so that the cells within the tubule segments produced a monolayer around both ends of the tubule segments. The samples were then incubated with terminal transferase reaction buffer. DNA fragments were 3'end-labeled with Dig-dd-UTP as described [167, 169]. Dig-dd-UTP was detected with the antidigoxigenin antibody conjugated with horseradish peroxidase and the antibody was localized by diaminobenzidine substrate. After light counterstaining with hematoxylin, the samples were dehydrated and mounted.

Immunohistochemistry for active caspase-3 (I)

Ovarian tissue was frozen, cut into cryosections, dried and fixed in acetone. The sections were then washed and blocked with blocking solution. Rabbit polyclonal antibody to active caspase-3 was added to the preparations in blocking reagent and incubation was performed overnight. After incubation, the slides were washed and the primary antibody was detected with a biotin-conjugated goat anti-rabbit IgG secondary antibody. The antibody was localized with diaminobenzidine substrate. After light counterstaining with hematoxylin, the samples were dehydrated and mounted.

Androgen receptor (AR) and Ki-67 immunohistochemistry (II)

Ovarian tissue samples were fixed in formalin, dehydrated and embedded in paraffin blocks. Serial sections of the tissue were deparaffinized, rehydrated and washed. They were then microwaved in citric acid for antigen retrieval, washed and blocked with blocking solution. Rabbit polyclonal antibody to AR or rabbit polyclonal antibody to Ki-67 was applied to the preparations, and incubation was carried out overnight. The rest of the protocol was carried out as described for immunohistochemistry for active caspase-3 (above).

Stage-specific seminiferous tubule preparations (III-IV)

Sixteen hours or 21 days after total-body irradiation, the testes were decapsulated in phosphate-buffered saline in a Petri dish. The seminiferous tubules were gently teased apart, and three 1-mm-long segments of seminiferous tubules at each of the stages II–V, VII–VIII, and IX–XI per mouse were prepared [216-218]. The different developmental stages of the epithelial cycle are distinguishable by their varying capacity to absorb light, so that the more advanced-stage spermatids there are within a seminiferous tubule segment, the darker it seems under a preparation microscope (Figure 5 A).

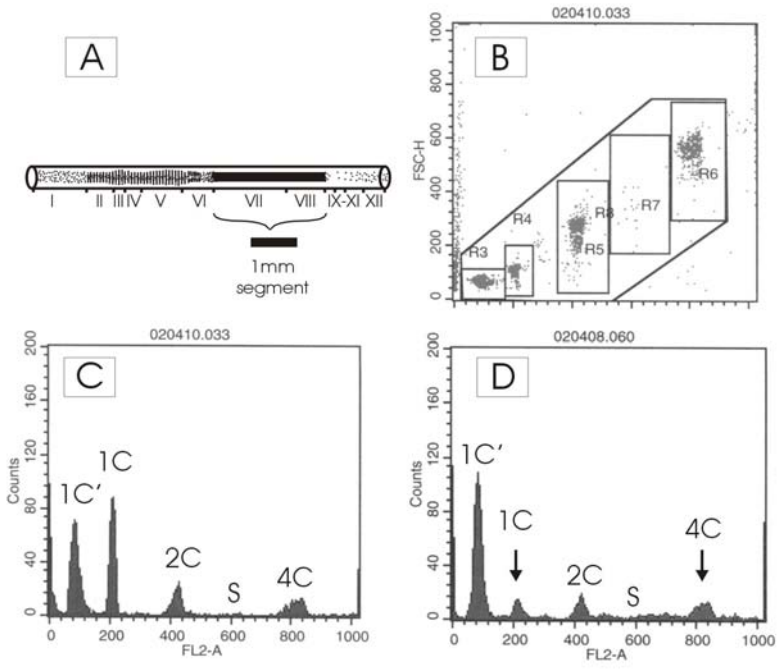

Figure 5. Evaluation of germ cell numbers before irradiation and 21 d after irradiation by flow cytometry. (**A**) 1 mm segments of seminiferous tubules of stages II-V, VII-VIII and IX-XI were cut based on the ability of each section to scatter light. Increased light absorption is associated with progressive chromatin condensation. Different stages are defined by Roman numerals. (**B**) Each nuclei (cell) population was gated out from plot charts in flow cytometry in order to count the relative cell numbers. Fluorescent beads used as internal volume standard were gated out from the chart. (**C**) DNA histogram of stages VII-VIII of the seminiferous epithelial cycle analyzed from unirradiated contol mice. Fluorescense intensity appears on the abscissa and the area of each peak reflects the number of nuclei (cells). The condensed step 13-16 spermatids forming the 1C' peak bind less propidium than step 1-12 spermatids forming the 1C peak. The 2C peak contains spermatogonia, preleptotene spermatocytes and somatic cells, the S-peak spermatogonia and preleptotene spermatocytes and the 4C peak primary spermatocytes and spermatogonia. (**D**) 21 d after a 0.5 Gy radiation dose a reduction in the number of nuclei (cells) can be observed in the 1C and 4C populations (arrows). R = gate number.

DNA flow cytometry (III-IV)

DNA flow cytometric analyses after irradiation were performed as described previously, with some modifications [218, 219]. Stage-specific 1 mm-long single segments of seminiferous tubules (Figure 5A) were treated with detergent in PBS containing bovine serum albumin and ribonuclease A. The nuclei were stained with propidium iodide, and diluted fluorescent particle solution was added to each sample as an internal volume standard for the quantification of absolute cell numbers. The samples were filtered and analyzed by a flow cytometer. A total of 5000 fluorescent impulses were counted excluding debris and the beads that were gated out of the rest of the sample (Figure 5 B). The numbers of nuclei in each peak of the DNA histograms were calculated and converted to absolute numbers by comparison to the internal bead standard. In the histograms, step 13-16 spermatids form the first, hypohaploid peak 1C' (Figure 5 C-D). These spermatids have condensed nuclei and therefore their fluorescence intensity is low. Step 1-12 spermatids are able to bind more propidium iodide and they therefore form the second peak, 1C. The 2C peak depicts the diploid amount of DNA. The 4C peak comprises mainly primary spermatocytes, excluding preleptotene spermatocytes, that are dispersed in the S-phase together with spermatogonia (G_2/M) [218].

Sperm analysis (III)

WT and ASMKO mice were sacrificed at the age of 20 weeks, sperm were collected from cauda epididymis and capacitated *in vitro* for 2 h in human tubal fluid medium. To measure both sperm concentration and motility, aliquots of semen samples were placed into a pre-warmed (37^{o}C) counting chamber. A minimum of 200 sperm from at least four different fields was analyzed for each specimen.

Measurement of ceramide and SM levels (III)

Levels of ceramide and SM were analysed as described previously [170]. Briefly, tissue from mouse testes was homogenized, centrifuged, and the supernatants collected for protein concentration measurements. Lipids were extracted and analyzed by the high performance thin-layer chromatography method as described previously [170]. Ceramide and SM concentrations were normalized to the total cell protein.

Data presentation and statistical analysis (I-IV)

All experiments were repeated on at least three independent occasions. Quantitative data represent low molecular weight DNA (optical density from x-ray films) from Southern blot analysis of apoptotic DNA fragmentation. In the experiment investigating nuclear apoptosis in the ovarian tissue, the 0 h time point was set at 1.0 (100%), and other time points (8 h, 24 h, and 48 h) compared with this. In the experiments for studying the effects of NAC and steroids on tissue survival, the 24-h time point cultured without NAC or steroid treatment was set at 1.0 (100% apoptosis) and the data from the samples cultured for one day and treated with different

concentrations of NAC were compared with this. Data obtained from 3 to 14 replicate experiments (mean ± SEM) were analyzed by a two-tailed paired t-test.

Comparisons between WT and ASMKO testicular lipid concentrations, testicular weights, DNA ladders, and sperm counts were performed by two tailed Student's t-tests, and stage-specific comparisons done by Mann-Whitney tests. Multiple comparisons between the lipids and testicular weights were done by 2-way ANOVA followed by two tailed t-tests. For the S1P experiments, a Mann-Whitney test was used to test differences in apoptotic cell counts between the S1P-treated and nontreated groups at the 16 h time point. Average counts were obtained from three to five squash preparations per testis and the amounts of apoptotic cells expressed as percentages of the total numbers of cells in the samples. At the 21 d time point, differences in cell numbers between the radiation dose groups within the three stages among the 1C and 4C populations were tested by one-way analysis of variance followed by a Dunnett test. The same method was used when testing the effect of S1P on cell numbers and for testing the effect of dose of radiation on testicular weight. One to three tubule segment measurements per stage per testis were used in the statistical analyses of cell numbers. The statistical analyses were performed in collaboration with a professional statistician. P-values < 0.05 were considered significant. Results are expressed as mean ± SEM.

RESULTS AND DISCUSSION

The Ovary

Human ovarian tissue survival in long-term cultures

Ovarian tissue cryopreservation is a promising method for fertility preservation. The tissue can be stored frozen for many years and it usually encompasses many primordial follicles that tolerate the freeze-thaw procedure well. Before the oocytes can be used for IVF-procedures, however, they have to be fully matured. If the tissue cannot be grafted back into the patient, as may be the case for many cancer survivors, *in vitro* maturation of the follicles is essential. The technique for *in vitro* maturation of primordial follicles may first require a phase in which follicles are cultured within the surrounding stroma. In the second phase, oocyte-granulosa cell complexes may be punctured from the tissue and matured further by IVM. Complete maturation of primordial follicles followed by successful IVF and birth of offspring has been so far achieved only in mice by a procedure beginning with organ culture followed by culture of isolated oocyte-granulosa cell complexes [1, 2]. Knowledge gained from *in vitro* cultures in animal studies cannot be directly extrapolated to humans, due to grave physiological differences (tissue structure, time needed for follicle growth, required growth factors) as well as different abilities of follicles of different species to endure severe stress. Attempts to establish long-term cultures for human ovarian tissue have resulted in follicle demise [30-33].

The aim of our first study was to investigate the mechanism of ovarian tissue cell death under long-term culture conditions and determine whether this cell death can be manipulated. We cultured small pieces of tissue for 1 and 3 weeks. The tissue pieces were placed in an extracellular matrix and the culture medium supplemented with human serum albumin (10%), FSH and insulin. FSH is not only essential for selectable follicles [24], but has also been shown to reduce atresia and promote follicle growth in cultured human ovarian tissue [33]. Insulin primarily affects glucose homeostasis, among other cellular effects [220], but also improves viability of follicles in long-term (2 weeks) human ovarian tissue cultures [125]. Despite our attempts to provide nutrient-rich culture conditions, the tissue survived poorly. After 1 week of culture, 36% of the follicles had become atretic and after 3 weeks the proportion had increased to 61% (Table 1 in I). Atretic follicles were identified by condensed granulosa cells and sometimes also by distracted granulosa cell layers, eosinophilic ooplasm, vacuoles in the ooplasm and shrinkage of the follicle (Figure 6). Atresia seemed to advance by initial condensation of the granulosa cells followed by condensation and eosinophilia of the oocyte, disappearance of the granulosa cells and eventual total atresia of the oocyte.

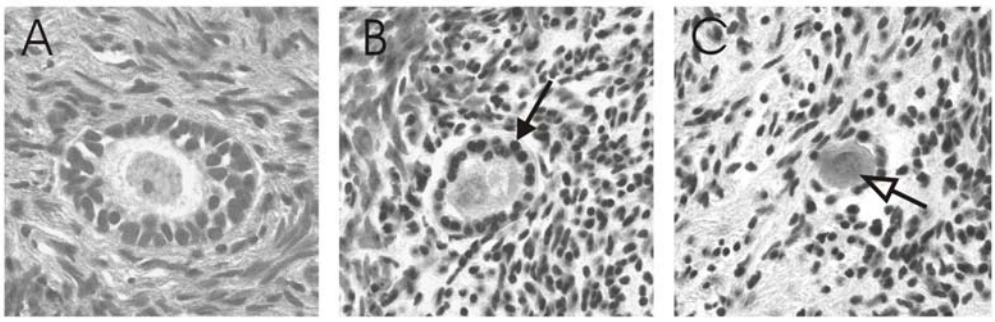

Figure 6. Ovarian tissue demise. (**A**) Normal human ovarian tissue and a healthy secondary follicle. (**B**) After culture for 1-3 weeks many follicles show signs of atresia, such as condensed granulosa cells (arrow), distracted granulosa cell layers, eosinophilic ooplasm, vacuoles in the ooplasm and shrinkage of the follicle. Many interstitial cells also condense. (**C**) In the final stages of atresia the follicle has lost most of its granulosa cells and the condensed oocyte (open arrow) persists in the tissue.

After culture, large areas of interstitial cells with condensed nuclei but also some with no visible cell structure were found. Controlled shrinkage of the nuclei points to apoptotic death pathways, whereas breakdown of the tissue structure suggests that some background necrosis also took place. Necrosis may be the consequence of too much apoptosis for the neighboring and phagocytotic cells to cope with. This is known to occur especially in cell cultures that lack cells specialized in phagocytosis [221]. The proportion of interstitial tissue with normal morphology had declined to approximately 50% in the 1-week cultures and to ~30% in the 3-week cultures. EM studies revealed clumping of the interstitial cell chromatin already two days after start of the culture. However, few abnormal granulosa cells were found at this time point and all of the oocytes seemed normal.

Taken together, despite the nutrient-rich culture conditions, the ovarian tissue survived poorly during the present *in vitro* culture. It seemed that the cells most susceptible to culture conditions were the interstitial cells. Their demise was followed by the granulosa cells and finally by the oocytes. This is a surprising finding as in a physiological situation apoptosis of immature follicles (primordial and primary) is normally driven by oocyte apoptosis and only in later developmental stages (preantral and antral) by granulosa cells [24, 76, 222]. Thus, it seems as if small follicles that are not physiologically destined for apoptosis face grave stress, such as suboptimal culture conditions, the follicle attempts to rescue the oocyte which ends up dying last. Another possibility is that oocyte apoptosis has less clear morphological apoptosis-like features and therefore its incipient degeneration is not as easily detected as that of somatic cells. However, the granulosa cells are closer to the dying interstitium than the oocytes and therefore possibly more prone to cell death. It is possible that the surrounding interstitial cells signal granulosa cells to initiate apoptosis. Although there are no previous studies on the ability of the stroma to induce follicle apoptosis in cultures, it clearly has a significant role in tissue demise *in vitro*. Additionally, the stroma is most likely needed for appropriate follicular development [24] and therefore is not dispensable. We conclude that it is of great importance to improve the survival of the stroma in long-term cultures.

Serum-free short-term human ovarian tissue cultures

We next created more defined tissue culture conditions from which serum and extracellular matrix were omitted and in which only a small amount of serum albumin was used (0.1%). In these kind of conditions the effects of specific components added to the culture medium can be studied without the interference of various undetermined agents. The tissue was cultured for up to 48 h, in which time an increasing amount of low molecular weight DNA fragmentation was detected by Southern blotting, although no clear morphological signs of apoptosis were apparent. Culture for 24 h increased DNA fragmentation in relation to that at 0 h ~3-fold (Figure 2 in I). DNA laddering mainly reflects the condition of the stroma, as in human ovarian biopsies the interstitial cells far outnumber any other type of ovarian cells. By ISEL we found that only very few follicles incorporated digoxigenin-dd-UTP, whereas considerable staining was found in the interstitium of the cultured tissue (Figure 3 in I). The interstitium contained areas of only apoptotic cells but also areas in which apoptotic cells were individually located among healthy cells. Immunohistochemical analysis of active caspase-3 revealed scattered interstitial cells with clear caspase-3 activation in the cultured tissue (Figure 3 in I). The granulosa cells very rarely showed any staining whereas some stained oocytes could be found. Very few ISEL positive cells or cells with active caspase-3 were detected in the fresh tissue. Therefore, we conclude that in the 24-hour serum-free cultures the process of interstitial cell apoptosis is rapid but that follicles mainly remain intact. Apoptosis seems to progress in a similar manner in both short- and long-term culture conditions.

Two aspects of caspase-3 expression deserve more attention. First, in the interstitial tissue only a small number of cells contained active caspase-3 whereas many ISEL positive and therefore most likely apoptotic cells were found. This discrepancy between caspase-3 and ISEL positive cells may be explained by the causality of these two factors; caspase-3 is an effector caspase in the apoptosis cascade and one of its functions is to activate DNase which cleaves DNA into the low molecular weight fragments detected by ISEL [223]. Caspase-3 expression is transient whereas DNA fragmentation is a late and more enduring event of cell death before the cell is dismantled completely. The second point of interest is that some caspase-3 expressing oocytes were found although they did not have any other signs of apoptosis. It has recently been found that caspase-3 is not required for murine oocyte apoptosis [105]. In mice, oocytes and granulosa cells utilize two distinct apoptosis pathways, one reliant on caspase-2 and the other on caspase-3 [105, 107]. Granulosa cells very rarely expressed caspase-3 in our *in vitro* cultures, which is in line with the observation that apoptosis is rarely initiated in this cell type.

Caspase-3 is a major executioner protease in many somatic cell types [224]. Theoretically, caspase inhibition by inclusion of caspase inhibitors (i.e. zVAD-fmk; benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone) in serum-starved culture medium could prevent caspase-mediated cell death, as has been done for example in cultures of fetal mouse ovaries [76]. However, our attempts to use zVAD-fmk to improve stromal tissue viability *in vitro* led to the switch of the mode of cell death from apoptosis to necrosis (unpublished results). This could be due to the fact that caspase activation is an important event in the apoptotic cascade and that upstream triggers cause cell death in the form of necrosis regardless of caspase inhibition.

N-acetyl-L-cysteine as an inhibior of human ovarian tissue apoptosis

Our novel short-term tissue culture model in which apoptosis proceeds rapidly allowed us to test the effects of potential anti-apoptotic/growth promoting factors without the influence of serum in the culture medium. Our second aim after clarifying the type of cell death in tissue cultures was to find components that could improve tissue survival. As *in vitro* cultures of ovarian tissue usually take place in normoxic conditions, where oxygen concentrations are considerably higher than those *in vivo*, oxidative stress may contribute to the atresia observed in cultures. Excessive production of ROS occurs when the fine balance between intracellular oxidants and their defense mechanisms are disturbed, and leads to apoptosis or necrosis [225, 226]. NAC is an antioxidant that works in the extracellular environment *per se* and a precursor of intracellular cysteine, thereby promoting glutathione synthesis [227-229]. We chose NAC as a potential inhibitor of apoptosis in cultures because (i) it has been shown to suppress apoptosis in cultured rat ovarian follicles [230] as well as in other gonadal cell types [214, 231, 232], (ii) NAC penetrates tissue very well *in vitro* and remains effective in cultures [214], (iii) it is a non-toxic compound with extensive clinical experience [233-235] and (iv) high levels of ROS have been shown to be detrimental to female and male fertility potential [236] indicating that oxidative stress is a major factor in female and male reproduction.

Addition of NAC to the ovarian tissue culture medium reduced apoptosis at concentrations of 25, 50 and 100 mmol/l. We were able to obtain up to a 32% inhibition at a concentration of 100 mmol/l as compared to samples cultured without NAC ($p < 0.05$) (Figure 5 in I). Thus, oxidative stress may play a role *in vitro* in human ovarian tissue apoptosis pathways that are triggered by suboptimal culture conditions. However, the exact mechanism underlying the death-suppressing action of NAC remains to be resolved. NAC encompasses an impressive array of potential targets of action other than those related to its antioxidative function. It is able to protect cells via mechanisms related to its nucleophility, and effects on DNA repair, mitochondria, gene expression, signal transduction pathways and metabolism (e.g. sphingolipid metabolism) [237, 238]. Additionally, high concentrations of NAC alter osmotic pressures which may affect cell death mechanisms. However, ovarian tissue most likely tolerates higher concentrations of NAC than individual cells because of its fibrous structure and several tightly bound overlapping cell layers that surround and protect the enclosed small oocytes. In fact, small concentrations may not be sufficient for adequate penetration of NAC into the tissue.

Based on our findings, NAC is a survival factor for human ovarian tissue during *in vitro* culture, which indicates that oxidative conditions are harmful in cultures. Thus, it can be assumed that a low-oxygen culture environment (e.g. 5% oxygen, 95% nitrogen) may be advantageous for the tissue. Low oxygen concentrations have previously been shown to be beneficial for cultured sheep preantral follicles [239]. Incubation of human ovarian tissue in low-oxygen incubators could theoretically be recommended. In this case exposure to high oxygen levels outside the incubator, i.e. during tissue handling, should be avoided to prevent multiple exposures to oxygen that could be even more harmful than continuous exposure. Therefore, incubation with NAC or other agents designed to protect against oxidative damage may be more practical and economical.

Expression of AR in human ovarian tissue

Based on previous *in vivo* and clinical studies [86, 87, 240, 241], androgens may have growth promoting and trophic effects on the ovaries and may therefore be good candidates for supporting ovarian stroma in cultures. For example, endogenously hyperandrogenic women (e.g. women with polycystic ovarian syndrome) and women with enforced hyperandrogenism (e.g. female-to-male transsexuals) have increased numbers of follicles at all developmental stages and characteristically extensive stromal hyperplasia [240-242].

We first investigated the distribution of ARs in the human ovarian cortex. We found some staining for AR protein in scattered stromal cells and in granulosa cells of primordial and primary stage follicles (Figure 1 in II). The fresh ovarian biopsies did not contain more advanged-stage follicles than primary follicles. AR immunoreactivity was located mainly in the nuclei of interstitial cells and granulosa cells, indicating that these cell types would be responsive to androgen treatment.

According to previous studies on AR expression in primates, AR is expressed in granulosa cells, the theca-interstitium and stroma [243-246]. These studies agree that AR is abundant in the granulosa cells of healthy growing follicles, especially at the preantral and early antral stages, but that expression is less distinct in the theca-interstitium and stroma. Our finding that ARs already exist in primordial follicle granulosa cells is novel. Although interspecies differences in the ovarian AR location may exist even between primates, the explanation is most likely methodological. However, pinpointing the exact methodological aspects needed for the detection of ARs in primordial follicle granulosa cells is difficult. The rabbit polyclonal antibody to a site near the N-terminal end of the human AR receptor (N-20 Santa Cruz, CA) showed specific staining also in testicular tissue used as a positive control, whereas conclusive results for antibodies against other sites of the receptor (epitopes localized within the C-19 terminal of the hormone binding domain or in the central DNA binding domain) were not achieved for the ovary or the testis (unpublished results).

Androgens are thought to be involved in regulating follicular responsiveness to FSH and thereby contribute to determining the follicle selected for ovulation [85]. Accordingly, androgen treatment of rhesus monkeys for 10 days has been shown to increase FSH receptor mRNA levels in all stages of follicular development, which has been thought to promote FSH action and the conversion of androgen to estrogen. Interestingly, treatment of rhesus monkeys for 2 days with recombinant FSH has also been shown to increase AR mRNA in primary follicles (but not in larger follicles), suggesting a potential mechanism by which FSH may support early follicular development [247]. Androgens may therefore promote growth of primate preantral follicles through ARs by amplifying the effects of FSH. This may in part explain the trophic effects of androgens on the ovaries. Androgen treatment of rhesus monkeys for up to 10 days has also resulted in a large increase in IGF-I and IGF-I receptor mRNA in primordial follicle oocytes and in increased numbers of primary follicles in the ovary. It has therefore been suggested that androgens trigger primordial follicle development through IGF-I signaling [87].

Effects of sex steroids on cell death

Although chronic hyperandrogenism in women is often associated with poor reproductive performance, short term treatment of rhesus monkeys with testosterone and DHT have been shown to stimulate follicular growth and reduce apoptosis resulting in improved survival of small follicles [86]. These two androgens were also shown to increase granulosa and theca cell proliferation as detected by Ki-67 antigen expression [86] and to recruit dormant primordial follicles [87]. The similarity of their effects indicated that androgen receptors mediate their actions and that the effects are not due to conversion of testosterone to estradiol [86, 87]. In addition to the AR-mediated and growth factor-like effects, steroid hormones have been suggested to take part also in the modulation of antioxidative defences [248] and may therefore participate in alleviating alterations in antioxidative homeostasis. Thus, we hypothesized that androgens could be beneficial for cultured human ovarian tissue.

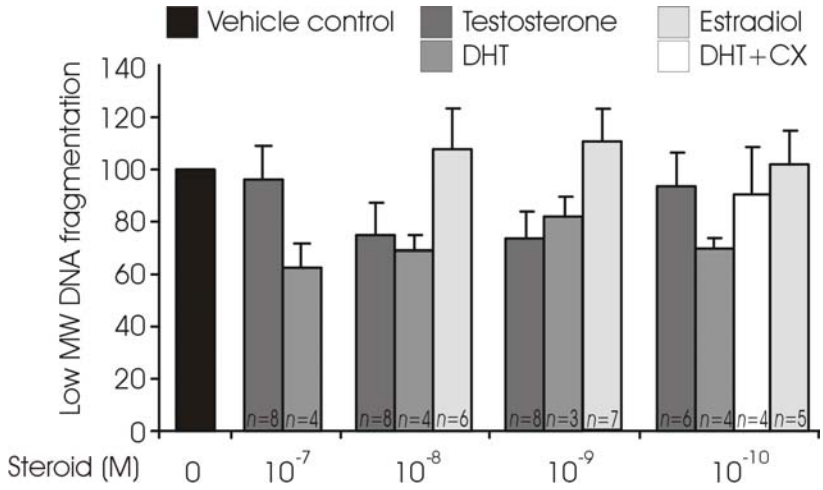

Figure 7. Inhibition of human ovarian tissue apoptosis by testosterone and DHT and lack of inhibition by 17β-estradiol. Slices of human ovarian tissue were cultured for 24 h under serum-free conditions in the absence or presence of testosterone, DHT and 17β-estradiol. DNA extracted from the tissue was 3'end-labeled with Dig-dd-UTP, electrophoresed, subjected to Southern blotting and analyzed and quantified as described in the "Materials and Methods". The digitized quantification of the low MW DNA fragments in the samples cultured in the absence of steroids was set a value of 100%, and the amount of low MW DNA fragments in the other samples were expressed in relation to this. An androgen receptor inhibitor (Casodex; CX) was applied to some of the cultures containing 10^{-10} M DHT. Each value represents the mean ± SEM of the indicated number of independent experiments.

To evaluate the effects of endogenous androgens on cultured human ovarian tissue, we studied the roles of testosterone and its non-aromatizable metabolite DHT on tissue survival (Figure 7). Testosterone suppressed *in vitro*-induced ovarian tissue apoptosis only marginally. At a concentration of 10^{-9} M the suppression was 26% ($p < 0.05$), as measured by the total amount of low molecular weight fragmentation in Southern blot analysis (Figure 3 in II). The effect of other

testosterone concentrations (10^{-7}, 10^{-8} M and 10^{-10} M) on apoptosis did not reach statistical significance. The concentration at which testosterone had slight suppressive effects, i.e. 10^{-9} M, is within the range of total serum testosterone in adult women, i.e. 0.5-2.5 x 10^{-9} M. However, most of the serum testosterone *in vivo* is bound to sex hormone binding globulin (SHBG), so that the amount of free testosterone is only 0-0.5 x 10^{-10} M. In women with hyperandrogenism and often also with concomitant polycystic ovary (PCO)-like histopathological changes in the ovary, serum free testosterone concentrations are frequently disproportionately higher than the total testosterone due to reduction of SHBG. In female-to-male transsexuals with normal ovaries, serum free testosterone levels have been shown to increase from 0.095 ± 0.052 x 10^{-10} M to 1.49 ± 0.46 x10^{-10} M after a 6-month treatment with testosterone [241]. Rhesus monkeys that received high-dose testosterone (4 mg/kg per day for 3 days) or lower dose testosterone (400 μg/kg per day for 10 days) manifested enhanced follicular growth and survival [86]. It is difficult to estimate how high a level of testosterone the ovaries and the enclosed follicles need to be exposed to *in vivo* in order to gain growth promoting and antiapoptotic effects. Therefore, our search for the right concentration for *in vitro* cultures was purely experimental.

In a previous study on rhesus monkeys, DHT treatment had identical effects to testosterone on the ovaries [86]. In our studies, DHT suppressed ovarian tissue apoptosis more effectively than testosterone (Figure 7). At most, a DHT concentration of 10^{-7} M suppressed apoptosis by 37% ($p < 0.05$) (Figure 4A in II). The statistically most significant anti-apoptotic effect was achieved by 10^{-10} M DHT, which reduced apoptosis by 30%. Both the DHT-treated and control tissue contained several apoptotic (detected by ISEL), but no proliferating (detected by immunohistochemistry for Ki-67) interstitial cells (Figure 4B in II). The suppressive effect of DHT was reversed by the AR antagonist Casodex at 10^{-6} M (Figure 4C in II), thereby suggesting that the effects of androgens are AR-mediated. Estradiol, at concentrations of 10^{-8}-10^{-10} M, did not suppress ovarian tissue apoptosis *in vitro* (Figure 5 in II and Figure 7).

Our results that testosterone and DHT suppress ovarian stromal tissue apoptosis *in vitro* and that the effects are, at least to some extent, mediated by ARs, are in an apparent discrepancy with the scarcity of ARs in the stroma. Although AR expression in the stroma was not abundant, it was nevertheless existent. It is possible that ARs are present in a much greater proportion of the interstitial cells, but only below detection levels. A further possibility is that another type of AR (beta?) resides in the tissue but cannot be detected by current methodology. Furthermore, other mechanisms not associated with ARs may contribute to the androgen-mediated inhibition of ovarian tissue apoptosis, e.g. antioxidant capacity [248, 249]. Our finding that testosterone is not as potent an inhibitor of apoptosis as DHT is in accordance with the fact that testosterone can be metabolized into estrogen, which again did not have any antiapoptotic effects. Previous studies on non-human primates have suggested that estrogens are inhibitory or inactive in regards to follicle development [250, 251].

Estradiol has an important role in the regulation of the hypothalamus-pituitary-gonadal axis. Estrogen receptors can also be found in the ovary. Estrogen receptor (ER) β can be found in granulosa cells from the primary stage, in the theca and in scattered interstitial cells. ERα is expressed in the interstitium, but granulosa cells do not express ERα until the follicles reach antral stages [243, 252]. Estrogens also have paracrine roles in the ovary, such as increasing gonadotropin receptor expression in the granulosa cells and enhancing gap junction formation between granulosa

cells [253]. However, the exact role of estrogens in oogenesis is unclear. A recent study on young aromatase KO mice, lacking the ability to synthesize endogenous estrogens, has revealed that estrogens are not essential for the production and growth of mature oocytes [254]. These young aromatase KO mice had elevated serum FSH and LH levels, but apparently healthy although anovulatory follicles in their ovaries. Following a standard superovulation protocol the oocytes did not ovulate. After manual recovery they nevertheless were able to fertilize and develop into blastocysts at the same rate as WT oocytes of littermates. The authors concluded that estrogen is required as a negative regulator of gonadotropin production for successful ovulation but it is not essential for the production of fertilizable oocytes capable of complete preimplantation development [254]. In the present study estradiol had no effect on ovarian tissue survival *in vitro*.

Androgens, especially DHT, seemed to be able to improve ovarian interstitial tissue viability in our cultures. However, we still needed to confirm that they would not be pro-apoptotic to developing granulosa cells. Excess immature granulosa cells collected from the aspiration fluid after oocyte retrieval for IVM and subsequent IVF stained positively for ARs. The staining was located in the cell nuclei (Figure 6A in II). The intensive immunostaining (i.e. high number of ARs) present in the IVM granulosa cells seems to have physiological relevance since these cells derive from the immature selectable pool of follicles. As discussed above, androgens are though to take part in modulating FSH responsiveness at intermediate stages of follicular development (preantral and early antral stages) and thereby contributing to determining which follicle will be selected for ovulation [247, 255]. As expected, culture of immature granulosa cells with 10^{-10} M DHT did not induce cell death (Figure 6C in II). Therefore, it can be postulated that DHT most likely is not pro-apoptotic to the developing follicles in the cultured ovarian tissue and is, therefore, a suitable component for ovarian tissue cultures.

Another alternative for prolonging ovarian tissue survival *in vitro* would be pharmacological intervention in apoptotic pathways. Prevention of early events in the interstitial tissue apoptosis cascades could be more fruitful than inhibiting late events, as inhibition of the late events of apoptosis may switch the mode of cell death to necrosis [76]. However, inhibiting early events in the apoptosis cascade would require additional research to clarify which events are crucial for cell demise in culture conditions. The role of apical apoptosis routes, including one involving ceramide, in ovarian tissue apoptosis remains to be clarified. Interestingly, ceramide and oxidative stress often seem to be intimately related in apoptosis induction [238]. A potential target for ceramide action are the mitochondria, where it induces Cyt c release and causes a decrease in mitochondrial transmembrane potential [66, 67]. In several types of cells ceramide synthesis can be attenuated by treatment with NAC [256-258].

The above studies were conducted in order to improve the survival of ovarian tissue in culture, which in turn is a prerequisite for achieving full maturation of human oocytes *in vitro*. Put into perspective, when the technical challenge of achieving *in vitro* maturation of human primordial follicles is overcome, a 4 mm ovarian biopsy from a 30-year-old woman would potentially yield approximately 120 small follicles, each theoretically capable of producing a mature gamete. This would be sufficient for a number of IVF treatment cycles (all that the woman would ever need) and spare oocytes could be donated to other patients undergoing IVF, or for research purposes [40, 259].

The testis

Response of the mouse testes to irradiation

Similarities exist in the process of spermatogenesis between different mammalian species [260]. Spermatogenesis is a strictly regulated cyclic process. Cohorts of germ cells enter the spermatogenic process at regular intervals so that associations of generations of cells are always found together and the stages follow each other in a well-defined order (Figure 8) [261]. Formation of the differentiating spermatogonia determines the species-specific duration of the epithelial cycle [203], which is approximately 9 days in mice [139] and 16 days in humans [262]. The cellular associations can be divided into stages of the epithelial cycle [203]. Stages are determined by the development of the spermatids: the morphology of the acrosome or by the elongated shape of the nucleus [139]. In the mouse 12 such stages can be distinguished and they follow each other consecutively so that a cross-section of a tubulus is always occupied by only one stage (Figure 8) [139, 262]. In humans there are 6 stages that follow each other spirally. A cross-section of a human tubule therefore always contains two or more stages [146, 262-264]. The duration of the spermatogenic process is approximately 35 days in mice [260] and 74-76 days in humans [265].

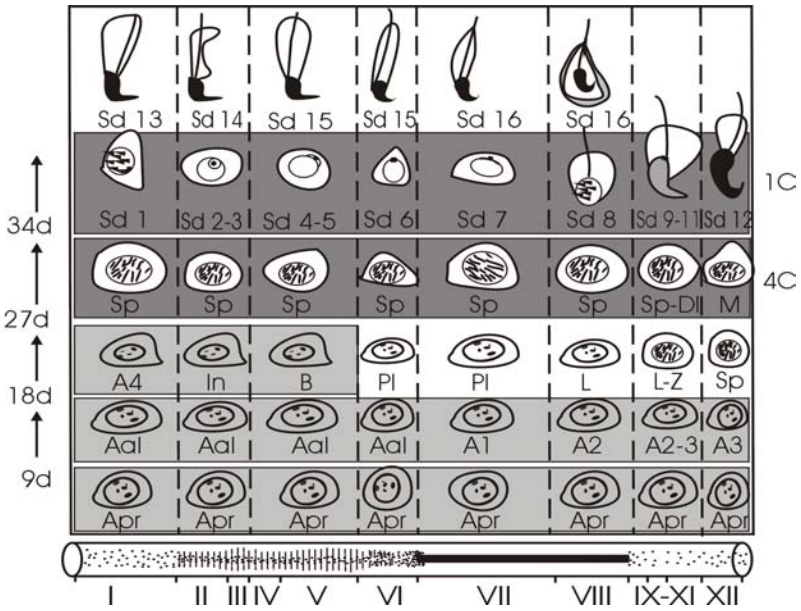

Figure 8. Seminiferous epithelial cycle in the mouse. Each of the 12 stages (Roman numerals) of the cycle contain specific cell associations. The duration of the cycle is constant, approximately 9 days, so that early spermatogonia differentiate into spermatocytes in 9 days and into spermatids in 18 days. Spermatogonia, the developmental stage most vulnerable to irradiation, are shown in light gray. Their demise can be detected 16 h after irradiation. 21 days after irradiation the death of spermatogonia at the time of irradiation can be detected as reduced numbers of spermatocytes (4C) and spermatids (1C), shown with a dark gray background. Apr, A-paired spermatogonia; Aal, A-aligned spermatogonia; A1-A4, type A1-A4 spermatogonia; In, intermediate speramtogonia; B, B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; Sp, spermatocytes; DI, diplotene spermatocytes; M, meiotically dividing spermatocytes; Sd 1-16, step 1-16 spermatids.

The biological events responsible for responses to external stresses should be essentially similar in mice and humans. In mice, irradiation seems to be especially harmful to the rapidly dividing and differentiating spermatogonia, as well as preleptotene spermatocytes [152, 266-269]. The radiation-induced spermatogonial degeneration has been identified to be apoptotic [153]. In humans later spermatocytes and spermatids also appear to be more resistant to irradiation than spermatogonia [270]. Humans appear to be much more sensitive than mice to the testicular effects when analyzed at a short interval, because in humans there appears to be a delay before surviving stem cells start to repopulate the tubules, whereas mouse stems cells start differentiation immediately. Additionally, some quantitative differences between different mouse strains seem to exist [267]. However, both species have comparable sensitivities when sufficient time is allowed for recovery [269]. In humans, doses as low as 1.5 Gy impair sperm production and doses exceeding 2-3 Gy cause irreversible germinal loss. Bone marrow transplantation patients who receive total body irradiation often experience severe germ cell damage [11].

In order to search for ways to protect male germ cells *in situ* from irradiation-induced apoptosis, it is essential to use an animal model as the studies are highly experimental. Therefore, knowledge of how the testis of the animal, in our studies the mouse, responds to irradiation is important. Thus, we first irradiated mice with different doses of radiation and studied germ cell death at two time points: 16 h and 21 d. It has previously been shown that rat testicular germ cell apoptosis can be detected 8 h after irradiation and the highest numbers of apoptotic germ cells are found at approximately 16 h, after which total apoptosis starts declining [271]. Mouse spermatogonia develop into spermatocytes and spermatids over 21 d. If spermatogonia die as a result of irradiation, the number of spermatocytes and spermatids is reduced after this period (Figure 8 and Figure 3 in IV).

We found that in mice the most common type of cell death 16 h after total body irradiation was apoptosis and it affected mainly the germ cells. Spermatogonia were most frequently dying of apoptosis, although apoptotic spermatocytes and spermatids were also encountered (Figure 2 A-C in IV). After a recovery period of 21 d testicular weights were found to decrease with increasing doses of radiation. After doses of 0.5, 1.0 and 2.0 Gy the mean testicular weights had decreased by 23%, 30% and 44%, respectively (Figure 1 in IV). The number of cells in spermatid (1C) and spermatocyte (4C) populations markedly decreased with increasing dose of irradiation (Figure 4 in IV and Table I in IV). The decrease was more prominent in the spermatid than the spermatocyte population and in stages II-V and VII-VIII than in stages IX-XI. For 1C a significant decrease in cell numbers was first observed after a dose of 0.1 Gy in stages II-V (31%, $p < 0.05$), a dose of 0.5 Gy in stages VII-VIII (75%, $p< 0.001$) and a dose of 2 Gy in stages IX-XI (38%, $p < 0.05$). For the 4C population significant reductions in cell numbers were observed after 1.0 Gy in stages II-V (74%, $p < 0.001$) and VII-VIII (73%, $p< 0.01$) and after 2.0 Gy in stages IX-XI (77%, $p < 0.05$).

These findings, in accordance with previous literature, showed that the higher the dose of irradiation, the more the cell numbers in the spermatid (1C) and spermatocyte (4C) populations decreased. Thus, flow cytometry is a valid method for quantification of irradiation-induced testicular apoptosis in mice. We found that cell numbers decreased more in the spermatid than in the spermatocyte population, possibly because of a larger number of primary spermatocytes (which after 21 d form the 1C population) than of differentiating spermatogonia (which form the 4C

population). In the 21-day period the spermatocytes divide several more times on their way to spermatids than do spermatogonia on their way to spermatocytes.

Exposure of mice to irradiation of 5 Gy has been previously shown to increase germ cell death significantly and even 0.5 Gy can induce marked changes. The changes are most prominent in B spermatogonia and early preleptotene spermatocytes [153]. The mouse epithelial cycle has been divided into two parts in respect to its radiosensitivity. Spermatogonial stem cells are highly sensitive to irradiation in stages III-VIII, whereas stages VIII-III are more resistant, suggesting that cells with different proliferative activities have different sensitivities [266]. Susceptibilities of spermatogonia vary according to their stage of development, so that in stages III-VII the D_0 value (mean lethal dose) is 0.4-0.7 Gy (most likely belonging to A-aligned to A1 spermatogonia), in stages XII-I the D_0 is 1.0 Gy (A-paired spermatogonia) and in stages IX-X the D_0 value is 2.2 Gy (A-single spermatogonia). Differentiating A1 to A3 and B spermatogonia are rather sensitive to irradiation (D_0 0.4-0.5 Gy) [272]. In accordance, our results showed significant reduction in cell numbers following a 21-day recovery period already after 0.1 Gy in stages II-V and after 0.5 Gy in stages VII-VIII. Stages IX-XI were most resistant, with significant reduction in germ cell numbers only after 2.0 Gy (Figure 4 in IV).

Role of ASM deficiency in vivo in testicular and germ cell development and physiological apoptosis

Understanding the targets of and pathways responsible for irradiation-induced germ cell apoptosis is essential when aiming to find effective protective treatments. ASM is an enzyme that belongs to a family of sphingomyelinases that catabolise membrane SM into ceramide [273, 274]. Deficient ASM activity is the cause of type A and B Niemann-Pick disease (NPD) in which impaired SM degradation leads to SM and cholesterol accumulation [275]. Cells received from NPD patients, as well as from mice lacking functional acid sphingomyelinase (acid sphingomyelinase knock-outs; ASMKO), have become useful tools when investigating ceramide-mediated signal transduction pathways.

ASMKO mice have no residual ASM activity and their phenotype is an intermediate of the more severe type A and the milder type B NPD [276, 277]. Homozygous ASMKO mice appear normal at birth, begin to express symptoms for neurologic disease including ataxia and mild tremors at about 2-4 months of age, and die at the age of 6-8 months [276, 278]. Their fecundity is affected since reduced fertility can be observed before the onset of neurological symptoms [277]. Therefore, lipid accumulation potentially causes severe physiological defects in the testis and harms germ cell development or function. Lack of ASM may also lead to impaired production of ceramide and thereby to defective male germ cell death. Ceramide, metabolized from membrane SM or synthetized *de novo*, acts as an apoptosis-inducing second messenger in many tissues and cell types [279]. The intracellular balance of S1P and ceramide has been proposed to determine whether the cell will survive or die [70]. Many cancer treatments, including irradiation, have been shown to cause ceramide accumulation [59].

In several cell types irradiation directly acts on the plasma membrane, activating ASM to generate ceramide [58]. ASM has been shown to be an important enzyme for physiological and pathological female germ cell death [3]. In a recent study ovaries from ASM-deficient mice

contained increased numbers of primordial follicles as well as significant ovarian hyperplasia, indicating defective normal apoptotic deletion of fetal oocytes. Germ cells in ASM-deficient fetal ovaries also resisted apoptosis induced by culture in serum deprivation conditions, and this could be entirely recapitulated by culturing fetal WT ovaries with increasing concentrations of S1P. In addition, S1P protected mature WT oocytes from apoptosis induced by the chemotherapeutic drug doxorubicin [3]. Most importantly, resistance to irradiation-induced apoptosis was achieved in WT oocytes *in vivo* when they were protected by S1P prior to irradiation [3].

Both female and male germ cells are susceptible to irradiation and infertility may follow cancer treatments. Previous findings that human testicular tissue produces increased amounts of ceramide when germ cell apoptosis is induced *in vitro* and that S1P suppresses this apoptosis (by 30%) [170], indicate that a pathway involving ceramide may function in male germ cell death. In order to study the role of the SM pathway in the testis, our aim was to investigate the role of ASM deficiency i) *in vivo* in testicular and germ cell development and physiological apoptosis, ii) *in vivo* in irradiation-induced apoptosis and iii) *in vitro* in serum deprivation-induced apoptosis.

We found that a prominent feature of the ASMKO mouse testes was high SM content as compared to WT mice at the age of 8 weeks ($p<0.001$) (Figure 1 in III), indicating that ASM is required for maintenance of normal SM levels in the testis. The level of ceramide was the same in both groups (Figure 1 in III) and can be considered the basal intratesticular level of ceramide. No significant differences were detected between WT and ASMKO testicular weights at the ages of 7 d, 21 d, 8 w and 20 w, although a tendency for heavier testes in the sexually mature ASMKO mice (8 w and 20 w) was observed (Figure 2 in III).

We then investigated whether physiological germ cell apoptosis is disrupted in the ASMKO mouse testes, thereby affecting sperm production. Around the age of 3 weeks a physiological apoptotic wave eliminates large numbers of spermatogonia and spermatocytes [140], most likely in order to maintain the correct number of maturing germ cells per Sertoli cell. Disturbance of the apoptotic wave causes abnormal spermatogenesis and sterility [140, 143, 144]. However, low molecular weight DNA fragmentation analysis (Southern blotting) revealed that ASMKO mice expressed more germ cell apoptosis at the age of 3 weeks than at the other ages investigated ($p<0.05$), indicating the presence of the first apoptotic wave (Figure 3A in III). Little germ cell apoptosis was detected at the age of 7 d, and at the age of 8 w the amount of apoptosis had again declined to a level comparable to that in the WT mice of the same age and remained at this basal level at the age of 20 weeks.

We were unable to detect any pathological lipid accumulation by histological examination of 7-day-old ASMKO testes. By 21 d small vesicles had appeared in Sertoli cell cytoplasm and in the interstitium (Figure 3B in III). As lipid-laden foam cells are a part of the etiology of ASM deficiency [277, 280, 281], we suspect that the observed vesicles are lipid accumulations. At 21 d large numbers of apoptotic spermatocytes typical for the physiological apoptotic wave also resided in the ASMKO testes (Figure 3B in III). The testes from 8-week-old sexually mature ASMKO mice contained more lipid accumulations within the seminiferous tubules in the Sertoli cells and outside the tubules in the interstitium (Figure 3C in III) and by 20 w the lipid vesicles had grown in size and had become more numerous (Figure 3D in III). In none of the ages investigated did we observe lipid accumulations in the spermatogonia, spermatocytes or spermatids. These results are in accordance

with Butler et al. (2002), who found lipid accumulation in the somatic testicular cells, but not in the germ cells of sexually mature ASMKO mice [277].

Sperm concentrations were not statistically different between the 20-w-old WT and ASMKO mice (Table 1 in III). However, the percentage of motile spermatozoa was much lower in the ASMKO mice than in the WTs ($p<0.001$), and the defect in ASMKO sperm motility was even greater when progressive motility was measured ($p<0.001$). Our results that ASMKO sperm motility is defective at the age of 5 months is in accordance with the results of Butler et al. (2002), who found that sperm concentrations were similar in the WT and ASMKO mice at the age of 6 months, but the rapid motility was reduced in the ASMKO sperm and there were greater numbers of static sperm. Butler et al. found that the etiology behind the poor sperm resulted from membrane lipid accumulation and a subsequent regulatory volume decrease defect in the developing ASMKO sperm. The excess lipid in the sperm plasma membranes resulted in bends and kinks that retarded sperm motility. Approximately half of the ASMKO sperm mitochondria were also damaged leading to reduced flagellar motion. This, together with the premature loss of the acrosome granule, which was often observed in the ASMKO sperm, contributed to the reduced fertility of the affected mice [277]. The regulatory volume decrease deficits may also explain why the developing germ cells seemed to lack intracytoplasmic lipid accumulations as observed by EM. The ASMKO Sertoli cells that express gross lipid accumulation may not be able to phagocytose residual bodies from mature spermatids [277], resulting in excess surface areas in the midpieces of sperm tails in late spermatids and spermatozoa.

Male and female gametes seem to differ in regards to the role of ASM in germ cell development and physiological apoptosis. Male germ cells most likely develop normally in the fetal period despite an ASM deficiency, as at day 7 post partum the testes exhibit normal testicular weights, morphology and amount of apoptosis. In striking contrast, ASM has been previously shown to be essential in generating death signals in the fetal female germ line. At day 4 post partum ASMKO ovaries have increased numbers of primordial follicles as well as significant ovarian hyperplasia, indicating defective normal apoptotic deletion of fetal oocytes [3]. In sexually mature males ASM did not prove important to physiological apoptosis. We observed equivalent numbers of apoptotic cells in ASMKO mice and age-matched WTs. Furthermore, although sperm motility was affected in the ASMKO mice, the sperm concentrations were not statistically different from those in WT mice, which also suggests normal control of germ cell numbers. ASM deficiency does not seem to affect physiological germ cell death of sexually mature ASMKO females either. Histomorphometric analysis have previously revealed that the differences in the number of oocytes in neonatal ovaries is preserved at the age of 42 d, when female mice achieve sexual maturity, indicating that the rate of apoptosis is preserved postnatally. However, ASM is no doubt important to induced germ cell apoptosis in females [3].

Role of ASM deficiency in vivo in irradiation-induced apoptosis

Compelling evidence has been presented on sphingomyelinase activation and generation of ceramide in several cell lines in response to ionizing radiation [58]. ASM deficiency renders many cell types resistant to irradiation. Lymphoblasts from patients with NPD are unable to respond to ionising radiation with ceramide generation and apoptosis [282]. Similar kind of defects in ceramide

generation and apoptotic response to irradiation have also been detected in the ASMKO mouse lung endothelium [282], in the central nervous system [283] and in fibroblasts obtained from ASMKO embryos [63]. ASMKO embryonic fibroblasts are also partially resistant to serum withdrawal. In contrast, ASMKO thymic cells are essentially sensitive to irradiation [282]. The resistance to apoptosis seems very stress-type specific and can often be overcome by administration of natural ceramide [63].

We found that 16 h after irradiation the SM levels did not differ from those of the corresponding non-irradiated mice of either genotype (Figures 1 and 4 in III). Surprisingly, 16 h after irradiation the ceramide levels were comparable to those in the non-irradiated testes in both groups (Figures 1 and 4 in III). Some studies suggest that ceramide is generated rapidly after irradiation, while others report elevation of ceramide as a late and prolonged response [58]. The response seems to be cell-type specific. In testicular tissue the injured cells proceed into apoptosis gradually so that in the rat the number of dying cells peaks approximately 16 hours after irradiation and apoptotic cells can still be detected after 42 hours [271]. Therefore, one could assume that the production of ceramide would remain elevated for several hours post irradiation. Since ceramide generation precedes the onset of apoptosis [170, 282], the optimum time to measure possible peak ceramide levels would be prior to the 16 h time point. Also, according to a previous study on mice, the number of abnormal spermatogonia reaches a peak 12 h after irradiation and then declines [153]. The 16 h time point may therefore be too late. Considering that spermatogonia comprise only approximately 3% of the total number of germ cells in the testis, the number of dying cells at any given time point is very small in relation to the total cell population in testicular tissue. Thus, the elevation of ceramide levels may constantly remain undetectably low, although existent. However, as ceramide levels were elevated after using a trigger of cell death other than irradiation (described later), it is also possible that ceramide does not have an important role in irradiation-induced male germ cell apoptosis.

Testicular weights at 16 h post irradiation were not different from the unirradiated testes in the WT or ASMKO mice. 21 d after irradiation, weights of the WT testes had decreased by approximately 20% compared to the non-irradiated testes ($p<0.01$) whereas no significant reduction in testicular weights could be observed in the ASMKO mice (Figure 5 in III). This also rendered the ASMKO testes heavier than WT testes after the recovery period. We found spermatogonia to be the main cell type that died after irradiation, although apoptotic spermatocytes and occasional spermatids were also observed (Figure 6A in III). No differences existed in the amount of apoptosis between the WT and the ASMKO testes at 16 h (Figure 6B in III) or in the number of cells in the 1C (consisting of early spermatids) and 4C (consisting mainly of spermatocytes) cell populations at 21 d (Figure 7 in III). Nevertheless a trend for slightly larger cell populations was detected at 21 d in the ASMKO testes, which could in part explain why the ASMKO mice on average seemed to have heavier testes than WT mice after the 21 d recovery period. ASMKO testes also contain lipid accumulations that are not affected by irradiation and therefore may contribute to the testicular weights at 21 d.

Role of ASM deficiency in vitro in culture-induced apoptosis

As mentioned earlier, the pathway chosen for cell death depends on the apoptotic stimulus. Therefore, we further studied the role of ASM in male germ cell apoptosis by using another cell death model, i.e. *in vitro* cultures. We cultured ASMKO and WT testicular tissue under serum- and hormone-free conditions. Culture of tubulus segments, rather than isolated germ cells, maintains the physiological interactions between the cells of seminiferous epithelium, thus mimicking the natural environment of the germ cells. In this *in vitro* model, apoptosis has previously been effectively induced in human seminiferous tubules in a time of 4 to 5 hours [164]. In our preliminary experiment we cultured WT mouse testis tissue for up to 96 h after which we performed Southern blotting of the apoptotic DNA. Apoptosis induction was seen after culture for 24 h and the ladder pattern became more prominent at 48 h. After 72 h the ladders started to become unclear. 96 hours of culture changed the pattern into a smear as necrosis took over (not shown). Thus, mouse testis tissue seems to proceed into apoptosis more slowly than human tissue in culture conditions. Based on these preliminary experiments, we chose to culture testis tissue from WT and ASMKO mice for 24 h and 48 h, i.e. when clear ladder patterns were discernable, for further experiments.

We then investigated the amounts of SM and ceramide in the WT and ASMKO tissue cultures. After culture for 24 and 48 h the level of SM remained constantly lower in the WT than in the ASMKO tissue ($p<0.001$) (Figure 8 in III). The ceramide levels increased markedly in culture in both WT and ASMKO tissue. At 24 h the increase was approximately 76% ($p<0.05$) in the WT tissue and approximately 73% ($p<0.05$) in the ASMKO tissue and the overall ceramide levels were similar in both groups. After culture for 48 h, the increase in ceramide was 76% in the WT tissue ($p<0.05$) and 77% in the ASMKO tissue ($p<0.01$) as compared to the uncultured tissue (Figure 8 in III). Interestingly, it seems that the ASMKO germ cells possess a means of producing high levels of ceramide other than through the ASM enzyme. Accordingly, it has been previously shown that WT and ASMKO spermatozoa exhibit a similar degree of SM degradation *in situ*, and therefore have sphingomyelinase activity distinct from the ASM enzyme [277]. This may be true for the earlier stages of spermatogenic cells as well.

In both groups, apoptosis increased markedly during culture, but there were no differences in the amount of cell death between the WT and ASMKO germ cells at any of the time points or in any of the stages, as investigated by low molecular weight DNA fragmentation and ISEL (Figures 9 and 10 in III). Although apoptotic cells of all cell types were identified, the culture conditions mainly seemed to affect spermatocytes and early spermatids and to a much lesser extent spermatogonia and late spermatids (Figure 10A in III). Apoptotic cells could be found in all stages investigated. This is in accordance with human testicular tissue in which germ cells in the later phases of differentiation (i.e. spermatocytes and early spermatids) are most sensitive to the *in vitro* conditions [164].

According to the results presented here (Table 2), ASM does not seem to be essential for irradiation-induced male germ cell death *in vivo* or *in vitro*-induced germ cell death and ceramide accumulation, but other apoptotic pathways may exist. Ceramide can be synthesized not only by ASM but also by NSM through the catabolism of SM [284, 285]. Both ASM and NSM activities can be identified in the testis [286]. ASMKO mice possess physiologic levels of NSM activity [276], and therefore it may participate in ASMKO germ cell apoptosis. It has been suggested that in

some cell types NSM is responsible for the response to ionising radiation [287, 288]. Glutathione, the most abundant thiol-containing cellular antioxidant [289], is an endogenous inhibitor of NSM [64]. The glutathione-dependent apoptosis pathway appears to be responsible for an early ceramide response [289]. In a variety of cells glutathione depletion occurs during irradiation-induced apoptosis and allows the action of NSM [58]. We have observed that injecting WT mouse testes with the glutathione precursor NAC before irradiation is unable to suppress germ cell death in the testis (unpublished results) (Figure 9). Therefore, it is possible that NSM does not have a profound effect on radiation-induced male germ cell apoptosis. However, NAC is able to reduce human germ cell apoptosis *in vitro* [214], emphasizing the importance of the stimulus to the pathway chosen for apoptotic cell death.

Table 2. Summary of the effects of ASM deficiency on the mouse testis as compared to the WT testis at the age of 8-10 w.

Physiological situation	**ASMKO**
Sphingomyelin content	high***
Ceramide content	equal
Testicular weights	equal
Sperm concentrations	equal
Sperm motility	low***
Progressive motility	low***
Irradiation-induced germ cell apoptosis	
Sphingomyelin content	high***
Ceramide content	equal
Testicular weights at 16 h	equal
Germ cell apoptosis at 16 h	equal
Testicular weights at 21 d	high*
Germ cell numbers at 21 d	low
Culture-induced germ cell apoptosis	
Sphingomyelin content at 24 h	high***
Ceramide content at 24 h	equal
Apoptosis at 24 h	equal
Sphingomyelin content at 48 h	high***
Ceramide content at 48 h	equal
Apoptosis at 48 h	equal

*p<0.05, ***p<0.001 vs. WT

Some pathological stimuli (e.g. drugs and irradiation) are known to activate the *de novo* pathway and lead to sustained elevation of ceramide in many cell types [59]. However, it was recently found that in cultures of human seminiferous tubules ceramide generation is not dependent on ASM or on the *de novo* pathway, which further suggests that *in vitro*-induced apoptosis requires NSM for ceramide accumulation [170]. These results are in accordance with the present study on mouse testicular tissue, in which ASM deficiency is unable to prevent ceramide accumulation or germ cell death *in vitro*.

Additionally, other factors may contribute to germ cell apoptosis observed in the ASMKO mice. Ceramide may, for example, be rapidly converted into sphingosine, and may therefore not be detectable after irradiation. Sphingosine has been reported to enhance apoptosis [290]. In addition,

other apoptosis pathways than those dependent on ceramide accumulation may be utilized. ASMKO mouse embryonic lymphoblasts are normally sensitive to apoptosis induced by staurosporine, a potent protein kinase inhibitor, but no ceramide is generated [63]. In the testis, the expression of p53 is high in the germ cells [173]. It seems to be involved in irradiation-induced death of the differentiating spermatogonia, but not in that of the more radioresistant stem spermatogonia [176]. Therefore, even different developmental stages of germ cells may proceed into apoptosis through different routes. ASM- and p53-mediated apoptosis appear to be two distinct and independent apoptotic pathways, where the SM pathway is considered to be membrane based while p53-dependent apoptosis appears to be secondary to DNA damage [282].

Taken together, ASM deficiency results in abnormally high intratesticular SM contents, lipid accumulation and defective sperm motility in sexually mature mice. These defects are most likely not due to inadequate physiological apoptosis. ASMKO mouse germ cells are able to respond as efficiently to induced apoptosis as WT germ cells. These results suggest that the physiological effects of ASM deficiency on the testes are due to lipid accumulation and that although this enzyme may participate in male germ cell apoptosis, it is not crucial or possibly even necessary for apoptosis, but other apoptosis pathways may be utilized.

Protection of mouse testes from irradiation-induced damage

Our final aim was to investigate the possibility of preventing irradiation-induced male germ cell death *in vivo* with S1P. As discussed earlier, the ability to control early intracellular events that signal apoptosis may potentially be important in protection from radiation-induced germ cell loss, as inhibiting late events in the apoptosis cascade may only redirect the cell death pathway from apoptosis to necrosis. The encouraging results obtained from female mice, that irradiation-induced oocyte loss could be completely prevented in adult WT mice by protecting the ovaries with S1P prior to irradiation [3], tempted us to investigate whether S1P could also be useful in protecting male germ cells from radiation-induced cell death. In human testicular tissue ceramide levels increase during culture-induced male germ cell apoptosis and S1P is able to partially inhibit this apoptosis. Thus, the SM pathway may have a role also in apoptosis induced by irradiation. We chose a dose of 0.5 Gy for the following experiments because at this dose significant reduction in the number of mouse germ cells could be detected, yet germ cell death was not overwhelmingly extensive so that modest effects of S1P could still be observed.

Sixteen hours after 0.5-Gy irradiation we found occasional apoptotic germ cells, mainly spermatogonia, both in control and in S1P-treated testicular tissue. EM or squash preparations did not reveal any morphological abnormalities brought about by S1P (Figures 2 D-E and 5B in IV). ISEL staining of apoptotic cells did not show statistically significant protection of germ cells in any of the stages (Figure 5A in IV). After 21 days, examination of the number of cells in the 1C (spermatids) and 4C (spermatocytes) populations, which were primary spermatocytes and spermatogonia, respectively, at the time of irradiation, revealed S1P-based protection in the 4C but not the 1C population (Figure 6 in IV). In the 4C population the number of cells in the non-protected group dropped significantly after a dose of 0.5 Gy as compared to the nonirradiated controls. In the S1P-protected group the number of cells did not significantly differ from the nonirradiated controls after 0.5 Gy. At stages II-V protection of the testes with 50 μM and 200 μM

S1P resulted in 16% and 34% higher total cell numbers, respectively, than in the irradiated vehicle-treated animals. At stages VII-VIII and IX-XI the effect was more clear. At stages VII-VIII the protection was 35% and 47%, and at stages IX-XI 40% and 38% with doses of 50 µM and 200 µM S1P (Figure 9). Using a high concentration of S1P (1000 µM) did not further improve germ cell protection (Figure 9).

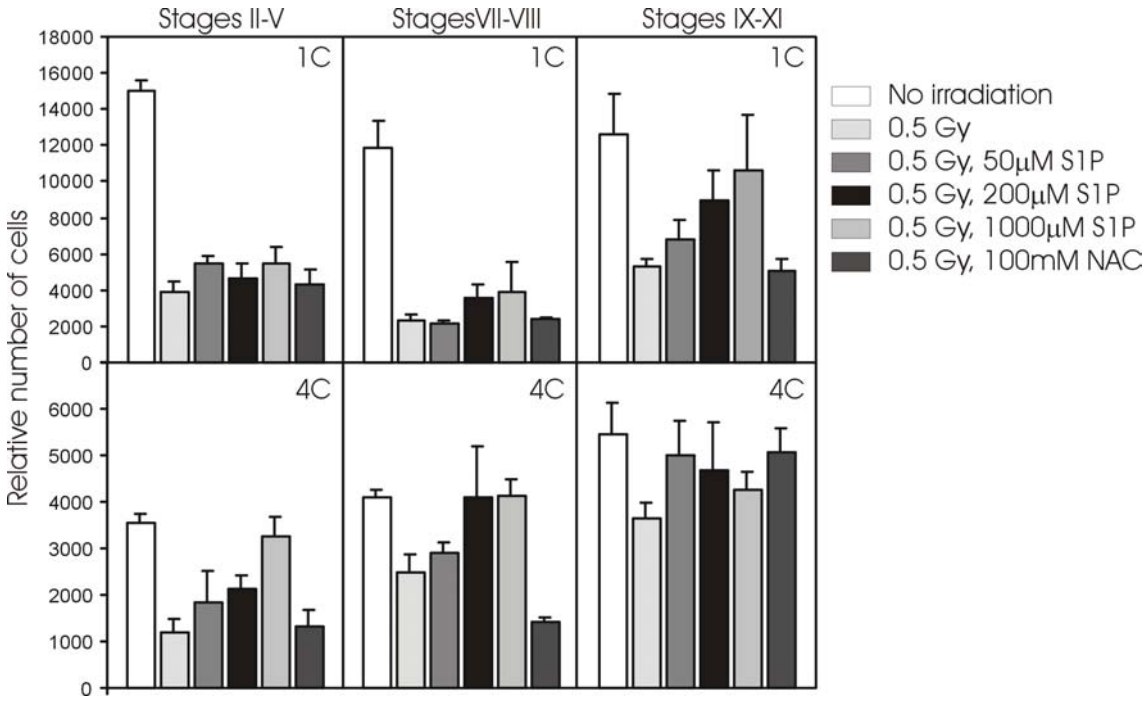

Figure 9. Number of spermatids (1C) and spermatocytes (4C) 21 days after S1P or NAC treatment and 0.5 Gy irradiation as measured by flow cytometry. Neither S1P nor NAC were able to confer protection to the germ cells in the 1C population, whereas S1P was able to confer moderate protection on the 4C population. Each value represents the mean number of cells in 4 testes from different animals ± SEM.

As presented above, the protection of differentiating spermatogonia by S1P was observed at 21 d but not at 16 h, although a trend toward lower numbers of ISEL-positive germ cells could be seen at 16 h. Physiological death of selected spermatogonia, spermatocytes and spermatids is a normal feature of spermatogenesis and the total number of apoptotic cells at the 16 h time point may have masked the protective effect of S1P on spermatogonia [144, 291]. Therefore, the protective effects of S1P were seen probably only at the 21 d time point.

The partially protective effect of S1P on germ cell apoptosis suggests that although S1P has a role in protecting male germ cells against irradiation, the SM pathway is neither the primary/initial or only pathway of male germ cell apoptosis, indicating the existence of alternative routes. This is in accordance with the germ cell death results obtained from the ASMKO mouse. The SM pathway and ceramide nevertheless may have a role in somatic testicular cells. In Sertoli cells, the SM hydrolysis pathway is involved in production of lactate, an energy substrate for germ cells [292]. In Leydig cells a ceramide-dependent pathway may be involved in regulating steroidogenesis [293] and possibly also apoptosis induction [294]. It is becoming evident that Sertoli cells are necessary

for providing the spermatogonia with optimal conditions for the complex maturation process [295, 296]. Sertoli cells and Leydig cells form an elaborate network of interactions with the germ cells within the seminiferous epithelium. Thus, the SM pathway may have a paracrine role in germ cell proliferation, differentiation and survival.

The mechanism by which S1P acts in the testis remains obscure. Although it has been described to act upstream in apoptosis cascades, it is not clear whether it acts upstream or downstream of DNA damage. S1P is a ligand for the G-protein-coupled $S1P_{1-5}$ receptors [72], but their potential roles in the regulation of male germ cell apoptosis remain to be unraveled. Our results suggest that S1P may have a role in inhibition of radiation-induced male germ cell apoptosis at the very early developmental stages, but may be less important for the later steps of spermatogenesis. Therefore, more work needs to be done before its therapeutic potential can be determined.

The present findings on ASMKO and S1P-protected mice suggest that male and female germ cell apoptosis induced by external stresses differ markedly from each other. Although ASM deficiency can protect female germ cells from apoptosis and S1P can protect WT oocytes from irradiation, this does not seem to be the case in males. Therefore, along with additional studies on the role of the SM pathway in male germ cell death, other apoptosis pathways should be studied in the search for potential therapeutics against pathological male germ cell death.

CONCLUSIONS AND FUTURE PROSPECTS

Previous studies attempting to mature human primordial follicles within cultures of ovarian cortical tissue have been unable to achieve complete *in vitro* development of healthy ovulatory follicle [30, 32, 33]. We found that survival of the cultured tissue and maturation of the enclosed follicles is hampered by apoptotic cell death, especially of the interstitial cells. This *in vitro* apoptosis of ovarian tissue could be partially suppressed by an antioxidant NAC and by the endogenous androgen DHT. Thus, addition of these components into the culture medium may improve ovarian tissue survival *in vitro*.

In future studies ovarian tissue culture conditions should be further improved and additional anti-apoptotic and growth promoting agents should be sought. However, the potential use of such compounds requires careful evaluation of the effects of these compounds on the developing follicles. As *in vitro* maturation technology rescues follicles that would otherwise most likely face atresia, the quality of oocytes becomes an issue of great concern when *in vitro* maturation of small follicles becomes feasible. Defects in maturation, such as improper imprinting, could manifest as embryonic death or excess birth defects [40]. Children born after IVF are essentially healthy [297], however, indicating that rescue of the selectable pool of follicles is rather safe. Additionally, inhibition of primordial follicle apoptosis by targeted mutagenesis of the pro-apoptotic *Bax* gene in mice does not increase pregnancy wastage [99]. However, *in vitro* conditions confer additional challenges to the oocytes. In the first study of complete *in vitro* maturation of mouse primordial follicles only one pup survived, but it was neither healthy or long-lived [1]. In an improved protocol several live pups were born and their health is under careful monitoring [2]. Therefore, much research lies ahead before *in vitro* maturation of human primordial follicles can be implemented in clinical service.

In the second part of this thesis project we aimed to investigate the effects of ASM deficiency in physiological and pathological germ cell apoptosis. We then attempted to protect the germ cells from irradiation-induced apoptosis with S1P. We found that ASM is not required for apoptosis in the testis, although it did seem necessary for maintenance of normal testicular SM levels and has a role in the production of normally motile sperm. Accordingly, attempts to protect mouse spermatogenesis with S1P from irradiation-induced apoptosis proved partly effective, protecting the very early stages of spermatogenesis but not the more developed stages. Different developmental stages of male germ cells may therefore proceed into apoptosis by utilizing different cell death pathways. As irradiation is especially harmful to spermatogonia, these cells should be the target developmental stage in the design of potential protective therapies.

The roles of ASM and S1P seem very different in males and females. In females ASM deficiency is able to suppress fetal oocyte apoptosis as well as *in vitro*-induced apoptosis, and S1P can completely protect the oocytes from irradiation-induced apoptosis [3]. As no discernible genomic damage is propagated to the offspring [135], this potential treatment has now proceeded to clinical development. However, more research needs to be carried out before sphingolipid pathways could be considered as therapeutic targets for male germ cell protection. Other, yet undiscovered, compounds could also be more effective in protecting male germ cells from irradiation. Persistent investigation of the apoptotic pathways in male germ cells therefore remains of utter importance. All potential antiapoptotic compounds require extensive animal studies before trials on humans, as

pharmacological inhibition of elimination of injured cells may cause tumors and transmit mutations to offspring. Use of human seminiferous tubule cultures may also provide useful information in view of species specificity. Only after extensive research could potential anti-apoptotic agents be used to benefit humans.

Much progress has lately occurred in human fertility preservation techniques. Very recently, transplantation of frozen-banked ovarian tissue underneath lower abdominal skin and subsequent percutaneous oocyte aspiration resulted in generation of a 4-cell embryo [35]. A significant break-through was achieved when another patient delivered a healthy baby after natural conception from orthotopically autotransplanted frozen-thawed ovarian tissue [34]. Both patients had had pieces of their ovarian cortex cryopreserved before sterilizing cancer treatments. Thus, many patients who have been previously considered infertile, e.g. those who have undergone chemotherapeutic treatments as well as Turner women, may get a chance to reproduce if their ovarian tissue is cryopreserved in time.

Much concern has arisen due to the lack of a clear plan of how to best utilize the banked tissue [298]. We also do not have a clear understanding of the consequences, such as the effects on the health of future offspring. A new proposal suggests autotransplantation of an intact ovary with its vascular pedicle in order to minimize post-transplantation ischaemic follicular demise [298]. However, whole organ cryopreservation remains technically extremely challenging.

Not only have females benefited from the recent advances in reproductive biology, but breakthroughs have also occurred in male reproduction. For example, Klinefelter men were thought to be infertile until the introduction of the ICSI-technique. Even when spermatozoa are not present in the ejaculate, surgical sperm retrieval directly from the testes has enabled the birth of several healthy infants [18]. However, an effective way to preserve immature germ cells, i.e. cryopreservation and transplantation of spermatogonia, has not yet proven successful in humans.

Today, science has enabled procedures that would have previously been perceived as unthinkable. The most recent developments include derivation of female and male gametes from mouse embryonic stem cells [299, 300]. Although no reports currently exist on the production of human gametes from embryonic stem cells, it has the potential in the future of becoming a source of gametes for donation to infertile couples.

Recent findings have also challenged old dogmas that have thus far controlled the modern way of thinking. Juvenile and adult mouse ovaries have been found to contain oogonial-like stem cells that continuously replenish the follicle pool [301]. These stem cells are mitotically active and possess a meiotic marker, both features which clearly distinguish them from adult oocytes [301]. However, the existence of mitotically active germ cells in the female ovary has also faced some criticism, pointing out that the results can also be interpreted differently [302]. If such germ cells do exist in the mouse ovary, they could be expected to be found also in the human ovary. If this was the case, harvesting the stem cells and creating new oocytes could become an intriguing option in future female fertility preservation, thus creating an infinite reserve of genetically own oocytes for individual use. However, at present this scenario is quite distant.

The above description of the recent achievements in reproductive biology highlights the dynamic nature and the fast pace of progress in reproductive sciences. Although much has been achieved, much remains to be done. New findings and ethical dilemmas constantly raise new questions and concerns.

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